UNIVERSITY OF LONDON THESIS

Degree MS Year 2007 Name of Author ELIZABETH RUTH DRYE

SPORADIC COLORECTAL CANCERS WITH MICROSATELLITE INSTABILITY: A STUDY OF VASCULAR ENDOTHELIAL GROWTH FACTOR

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JANUARY 2007

Presented for the award of Master of Surgery (MS)

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ABSTRACT

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Microsatellite high (MSI-H) tumours carry a better prognosis than microsatellite low (MSI-L) or stable (MSS) tumours for the reasons that have yet to be fully determined. Prognosis in CRC is associated with the degree of invasion of the primary tumour through the bowel wall and its spread there after. Such processes are governed by the Vascular endothelial growth factor (VEGF) family of cytokines, and through angiogenesis (via VEGF and lymphangiogenesis (via $VEGF_C$). Little is known however, of the relationships of VEGF and $VEGF_C$ in CRC with MSI, which is the aim of this thesis.

DNA and total cellular RNA were extracted from wax-embedded samples of CRC. MSI status was determined from extracted DNA by SSCP, examining loci BAT25, BAT26, BAT40, D5S346, D2S123, and D17S250;unstable loci, ≥30-40% (MSI-H), <30-40% (MSI-L), 0% (MSS). From extracted RNA transcripts of $VEGF_A$ isoforms $VEGF_{121}$, $VEGF_{165}$, and $VEGF_C$ were amplified by RT-PCR. The results were analysed by scanning densitometry, measured as total integrated optical density (IOD), and the levels of gene transcription determined by ratio to housekeeping gene, GAPDH-3. Angiogenesis was determined by examining blood vessel density using immunohistochemistry and antibodies to CD34. Lymphangiogenesis was determined immunohistochemically by $VEGF_C$ protein staining.

Sixty seven sporadic CRC were analysed [35 males (median 70 (range, 38-100) years), 32 females (77(53-91)) years], and 8 normal colon samples [5 males (66 (57-82) years), 3 females (71 (45-87) years]. MSI-H CRC were more in females (83%, $p<0.02$) and in the right colon (75%, $p<0.04$). $VEGF_{121}$ transcription was elevated in all cancers

compared to normal colon and was highest in MSI-L tumours (MSI-L 0.679, MSS 0.538, MSI-H 0.409, Normal 0.331, $p<0.01$). $VEGF_{165}$ transcription was reduced in all cancers compared to normal colon (MSS 0.491, MSI-L 0.428, MSI-H 0.413, Normal 0.592, $p>0.05$) and there was no correlation with MSI ($p>0.05$). There was a weak inverse correlation between $VEGF_{121}$ and Duke's stage (A 0.713, B 0.570, C 0.510, D 0.451, $p<0.09$) but there was no correlation with either $VEGF_{165}$ or $VEGF_C$ gene transcription. However, there was elevated levels of $VEGF_C$ protein expression in Duke's C and D tumours (A 59.6%, B 78.4%, C 80.0%, D 81.0%, $p<0.07$). $VEGF_{121}$ and $VEGF_C$ were elevated in MSI-L tumours (with a single dinucleotide marker mutated) compared to MSI-H tumours ($VEGF_{121}$ – MSI-L 0.679, MSI-H 0.409, $VEGF_C$ – MSI-L 0.542, MSI-H 0.471, $p<0.05$) and there was an inverse correlation of blood vessel density to MSI status (MSS 12.5, MSI-L 11.1, MSI-H 10.1, $p<0.09$).

Overall, this thesis demonstrates that wax embedded samples are a useful resource of CRC to investigate both gene transcription and protein expression and helps to conclude that MSI-H tumours have reduced angiogenic/lymphogenic potential whilst transcription of $VEGF_{121}$ may be important in the early growth and spread of CRC. Elevated $VEGF_{121}$ and $VEGF_C$ transcription with a singular di-nucleotide mutation supports the theory of a distinct MSI-L subgroup.

ACKNOWLEDGEMENTS

ACKNOWLEGEMENTS

To Dr Simon Hollingsworth for his support, guidance and encouragement, that I will complete this MS. To Professor Paul Boulos for his helpful pointers and encouragement. To Mr Garth Powell for his helpful discussions and technical advice. To Mr Samson Tou for his advice and discussions in learning the technique of PCR.

STATEMENT OF ORIGINALITY

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The RT-PCR, H&E and immunohistochemistry presented in this thesis is the sole work of the author except for the Duke's stage and MSI staging of the tumours. The Duke's stage was classified by the Histopathology Department at The Whittington Hospital NHS Trust, Highgate Hill, London, N19 5NF. The MSI staging of the colorectal tumourswas classified by Dr Siobhan SenGupta, member of the Colorectal Unit in the Department of Surgery at Royal Free and University College Medical School.

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ABBREVIATIONS

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ABC	Avidin:Biotin enzyme complex
APC	Adenomatous polyposis coli tumour suppressor gene
bp	Base pair
Bax	Bcl2-associated X protein
COX	Cyclo oxygenase
CRC	Colorectal cancer
DAB	3,3' Diaminobenzidine
DNA	Deoxyribonucleic Acid
5'	Five-prime
FAP	Familial Adenomatous Polyposis
GAP 3	Glyceraldehyde 3 phosphate dehydrogenase
GTP	Guanidine Triphosphate
H&E	Haematoxylin and Eosin
HNPPC	Hereditary Non Polyposis Colorectal Cancer
Ig	Immumoglobulin
Kras	Kirsten rat sarcoma 2 viral oncogene homologue
IOD	Integrated Optical Density
LOH	Loss of heterogenecity
MLH1	Mut L homologue
MMR	Mismatch repair protein
MSH2	Mut S homologue
MSI	Microsatellite instability
MSS	Microsatellite stable

MSI-L	Microsatellite low
MSI-H	Microsatellite high
p	Long arm of chromosome
p53	Protein 53 tumour suppressor gene
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
q	Short arm of chromosome
RNA	Ribonucleic Acid
RT-PCR	Reverse transcription
SMAD	Sma Mothers against decapentaplegic
Taq	*Thermus aquaticus*
TCR-4	Ternary complex factor four
3'	Three prime
TGFβRII	Transforming growth factor β receptor type two
VEGF	Vascular Endothelial Growth Factor
WNT	Wingless Int-1

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CHAPTER 1

INTRODUCTION

CHAPTER 1

1.1 Colorectal Cancer

The wealth of information gained to date on the epidemiology of colorectal cancer (CRC) is vast and beyond the scope of this introduction, however, a brief summary is presented below. A more expansive review can be gained from Keighley & Williams (1997).

1.1.1 Epidemiology

CRC is the second most common cause of death from cancer, in both males and females and alone was responsible for over 14,000 deaths in England, in 2000 (Quinn *et al.*, 2001).

Geographical Distribution

The incidence of CRC is not uniformly distributed among different populations. The highest incidence tends to be observed in urban populations of well educated individuals with high income levels such as Western Europe and North America. It is lower in less affluent communities of countries such as Asia, Africa, and South America (Blot *et al.*, 1976). However, various studies have demonstrated that rectal cancer shows less inter-population variation compared to colon cancer.

Age and sex

The incidence of CRC increases with age, with rectal and colon cancer developing mostly in patients between the ages of 60 and 69 years (Goligher, 1941 1984, Chu *et al.*, 1994). Only 2-4% of the population below the age of 40 years develop CRC. In this

age group there appears to be an association between gender and site of the cancer as tumours are predominantly in the right colon in males, and in the left colon in females. Overall, there is a fairly equal gender distribution in a ratio of females to males of 3:2 in colon cancer and of 7:8 in rectal cancer (Silverberg, 1981). In the younger population (<40 years), gender distribution is equal, while in older patients (>70 years) these cancers are more frequent in males (Umpleby *et al.*, 1984).

Site

There is an equal cancer distribution in the upper, middle and lower rectum (Dukes, 1940, Goligher, 1941). While in colon cancer, 50% are in the sigmoid colon, 25% in the caecum and ascending colon and 25% are in the transverse colon. Interestingly, since the late 1960's, an increase in the incidence of right colon cancer has been noted (Meigs *et al.*, 1977, Loffeld *et al.*, 1996).

Synchronous Cancer

Synchronous cancers are defined as distant tumours which are detected simultaneously, or diagnosed within one to six months from initial diagnosis, in this way, they are differentiated from metachronous cancers. Approximately 68% of synchronous neoplasms are found within surgical specimens, although with the increasing use of colonoscopy, either preoperatively or within 6 months following surgery, more neoplasms have been demonstrated in the rest of the colon (Reilly *et al.*, 1982, Langevin & Nivatvongs, 1984). Stage for stage, tumour survival is the same for both synchronous and solitary tumours (Passman *et al.*, 1996).

1.1.2 Classification of Colorectal Cancer

There are four macroscopic types of CRC;

- Ulcerative cancer - malignant ulcers with raised rolled edges and a necrotic base
- Polypoid cancers - proliferative and protruding from the bowel lumen
- Annular/stenosing - circumferential with an ulcerated surface
- Diffuse infiltrative - infiltrates the bowel for at least 5-8cm.

Microscopically CRC are heterogeneous in morphology, and are divided into well differentiated, moderately differentiated and poorly differentiated (Grinnell, 1939). Grade I, well differentiated tumours, resemble adenomas but active epithelial proliferation is present and the malignant component breaches the muscularis mucosa. Grade II, moderately differentiated, the malignant cells are more crowded together but are still arranged in a relatively regular glandular pattern. The walls of the glands are one or two layers thick and the nuclei stain deeply and irregular mitotic figures are present. Grade III, poorly differentiated, have at best cells arranged in irregularly folded rings, 2 or 3 rows deep around spaces or as a solid clump. The mitotic figures are frequent and at worst, there are very anaplastic cells which form sheets with no attempt to form glandular structures. In colloid or mucinous tumours the degree of differentiation varies and these tumours produce lots of mucin, which occupies at least 60% of the tumour volume.

Staging

The Duke's classification was originally described for rectal cancer only (Dukes, 1930 & 1940). Duke's A cancer is a growth limited to the wall of the rectum with no

extension to extrarectal tissue and no metastasis in lymph nodes. Duke's B is a growth that extends through the wall to the extrarectal tissue but the lymph nodes are free of tumour. In Duke's C the lymph nodes contain tumour.

In 1939 the classification was applied to colon cancer as well and was modified by Kirklin *et al.* (1949) to; A, a tumour which has not penetrated the muscularis mucosae, and B, a tumour which penetrates superficially or deeply into the rectal wall. Astler-Coller (1954) modified the B classification to incomplete or complete penetration of muscularis propria. Turnbull *et al.,* (1967) then added Duke's D classification to include tumours with extensive spread beyond the bowel to other organs.

A working party on staging agreed that the pathologist should describe a tumour by the following characteristics (UKCCCR, 1997).

1. Gross description
    a) Distance of upper edge of growth from proximal margin and lower edge of growth from distal margin (cm)
    b) Axial and transverse diameters of growth (cm)
    c) Circumference of bowel at mid point of tumour (cm)
    d) Appearance of tumour (ulcerating, protuberant, diffusely infiltrating, perforated)
2. Local spread
    a) Spread through tissues of bowel wall
        i) Cannot be assessed (previous local excision or radiotherapy)
        ii) Neoplastic process limited to mucosa

iii) Limited to submucosa

iv) Limited to muscularis propria

v) Beyond muscularis propria but not penetrating peritoneum or invading adjacent organs

vi) Penetrates peritoneum, and/or

vii) Invades adjacent organs

b) Extent of spread beyond bowel wall (mm) and clearance at deep excision margin (mm) to be measured on gross specimen and confirmed histologically

c) Involvement of proximal and distal margins of specimen requires histological confirmation only when the margins are close to the tumour (<5 cm) or tumour is highly infiltrative

d) State whether tumour excision is complete or incomplete

3. Lymphatic Spread

a) Number of nodes draining bowel segment harbouring tumours

b) Number of nodes with metastatic cancer

c) Metastasis in apical node (immediately below vessel ligature)

d) Extramural tumour deposits apparently not within nodes

e) Lymphatic or perineural permeation

4. Venous Spread

Invasion of extramural veins

5. Histology

a) Type (adenoma, mucinous adenocarcinoma, signet ring cell carcinoma, other)

b) Differentiation (poor or other)

c) Invasive margin (expanding or infiltrating)

d) Peritumoural lymphocytes

6. Other pathology

a) Synchronous carcinoma

b) Adenoma

c) Other

1.2 Current theories for the molecular basis of Colorectal Cancer

1.2.1 Genetic principles of carcinogenesis

Carcinogenesis involves interference in several different processes which contribute to cell proliferation and formation of new blood vessels. These processes are essential for maintenance of a normal cell and include cell signalling, cell cycle regulation, DNA repair and genetic stability, telomerases, apoptosis, cell adhesion, angiogenesis and lymphogenesis (Alison, 2001).

Cancer cells develop by the acquisition of genetic information or by the loss of control of cell proliferation and cell death. Mutations in cells can produce oncogenes which are proteins that have gained a function, in contrast mutations of tumour suppressor genes lead to a loss of function (Bishop & Weinberg, 1996).

Gene Expression

Gene modification results in either qualitative or quantitative changes in gene expression. Alterations in the 5'upstream regulatory sequences cause the alteration of gene transcription whereas mutations in the 3' non coding regions increases the half life of mRNA. Quantitative changes in gene transcription can occur by amplification of a gene seen in advanced cancers or by altering the regulation of the gene seen in both early and advanced cancers. Altered regulation can occur by rearrangement of chromosomes resulting in the coding sequence of an oncogene under the influence of a strong regulatory/promoter sequence of another gene, a promoter insertion. Separately, genes can be amplified by chromosomal instability and multiple duplications of specific

regions of DNA when there are defective start signals at DNA replication forks (King, 2000).

Mutation

Qualitative changes in gene expression can occur by essentially two routes: a gene of normal size but altered activity, or a gene of abnormal size. Alteration in gene activity can also occur by a point mutation. For example, the proto-oncogene Ras is a GTP binding protein with GTPase activity that is important in signal transduction between cell membranes and the nucleus. When a point mutation occurs, most commonly at codon 3, 12, 13, or 61, ras GTPase activity decreases and so Ras activity is altered and in turn signal transduction to the nucleus as well. Abnormal sized genes in contrast are produced by chromosomal translocation. For example in acute promyelocytic leukaemia the gene for retinoic acid receptor α (RARA) normally located on chromosome 17 becomes translocated next to the promyelocytic leukaemia gene (PML) on chromosome 15. The abnormal but active protein PML-RARA is then produced by the fusion of the partial products from both genes (King, 2000).

Suppression

Repressor, or tumour suppressor genes are required for normal cell growth and function. The gene products are the tumour suppressor proteins which inhibit cell function by complexing with other effector proteins to block their action. As a result cell growth is inhibited by preventing cell cycle progression. As such, for tumour growth to occur genes which code for inhibitory proteins must be reversibly inactivated. Loss of tumour suppressor gene function occurs by the loss of one or both alleles depending on the specific gene, the end result being the loss of a functional protein and so uncontrolled

growth occurs. Tumour suppressor genes are inhibited by at least three different mechanisms;

- Phosphorylation of the protein
- Mutation which causes the loss or alteration of the protein
- Inactivation of the protein by other proteins.

Apoptosis

Programmed cell death or apoptosis is a tightly regulated process and the ability of tumour cells to avoid it contribute to cells with DNA damage not being diverted to apoptosis and so cells with damaged DNA persist increasing the possibility of malignant transformation and so the development of a tumour (Kerr *et al.*, 1972). Pro-apoptotic signals can arise from proteins such as p53 and bax and mutation of p53 is seen in more than 50% of cancers (Harris, 1996). In contrast inhibitory signals to prevent apoptosis originate from Bcl-2 proteins and over expression of Bcl-2 by chromosome translocation has been shown to have anti apoptotic activity (Vaux *et al.*, 1988).

Cell Signalling

Alteration in the mechanism of cell signalling can contribute to malignant transformation. Cancer cells are under the influence of both paracrine and endocrine but in addition to autocrine signalling. Cancer cells both produce the signals and then respond to their own signal by having the required receptors within the cell (Hanrahan & Weinberg, 2000).

Cell Cycle

Regulation of the cell cycle can also be altered when a cell undergoes malignant transformation. Mutational activation of the components of the cell cycle lead to signalling without need for receptors. In addition to mutational activation alterations to cell cycle inhibitors can lead to a loss of control of the cell cycle. Examples are retinoblastoma protein (pRB) and p53, which when inhibited contribute to tumour development.

Mismatch Repair

Normal cells have the ability to recognise damaged DNA and repair it. However if the mechanisms of recognition and repair are damaged, genetic instability develops leading to an increase in alterations within the genome. For example, failure to recognise and repair genomic errors occurs with errors in the mismatch repair (MMR) genes and this is responsible for the development of HNPCC. This is discussed in detail in Chapter 1.2.3. MMR genes normally recognise and repair errors in microsatellite regions of DNA sequences. When mutated they are no longer able to do this so errors remain within the DNA sequence and contribute to the development of cancer cells (Allison, 2001).

Hayflick Limit

The majority of normal cells have a defined life span limiting the possible number of times cells can divide, known as the 'Hayflick limit'. The life span of a cell is partly determined by telomeres which are protective caps at the ends of chromosomes used as primer points in DNA replication. With each replication of the DNA sequence the length of the telomeres get shortened until finally cells which have no telomeres are

recognised as damaged and diverted to apoptise. The majority of cancer cells develop in cells which have no telomerases but which have escaped cell death by reactivating the enzyme (Hayflick, 1997).

Cell Adhesion

Tumour development is also associated with altered expression of cell adhesion molecules. The loss of cell adhesion involves the loss of cell attachment to the basement membrane, effectively enabling invasion. Alternatively cell adhesion is increased allowing tumour cells to attach to endothelial cells, such as occurs in malignant melanoma (Christofori & Semb, 1999).

Colon cancer originates in the mucosal lining and develops with genetic changes. These changes in the mucosal layer provide an example of how alterations in genes lead to malignant transformation. There are two major pathways which result in the development of sporadic CRC; either by chromosomal instability (CIN), or by microsatellite instability (MSI). More recently a third pathway has been suggested which is microsatellite and chromosomal stable (MACS).

1.2.2 Classical pathway of carcinogenesis

Fearon & Vogelstein, (1990) hypothesised that CRC develops by mutational activation of oncogenes and mutational inactivation of tumour suppressor genes. They concluded that there needs to be mutations in four or five genes for the formation of a malignant tumour. The total accumulation of mutations rather than the order in which mutations occur are the most important. Mutations in tumour suppressor genes appear to cause phenotypic changes even when present in a heterozygous state. This results in the

normal colonic mucosa developing into a polyp with mutations of genes such as APC, COX-2 or K-Ras and with further mutations, for example p53 or 18q, the polyp will eventually develop into a carcinoma (Figure 1.1).

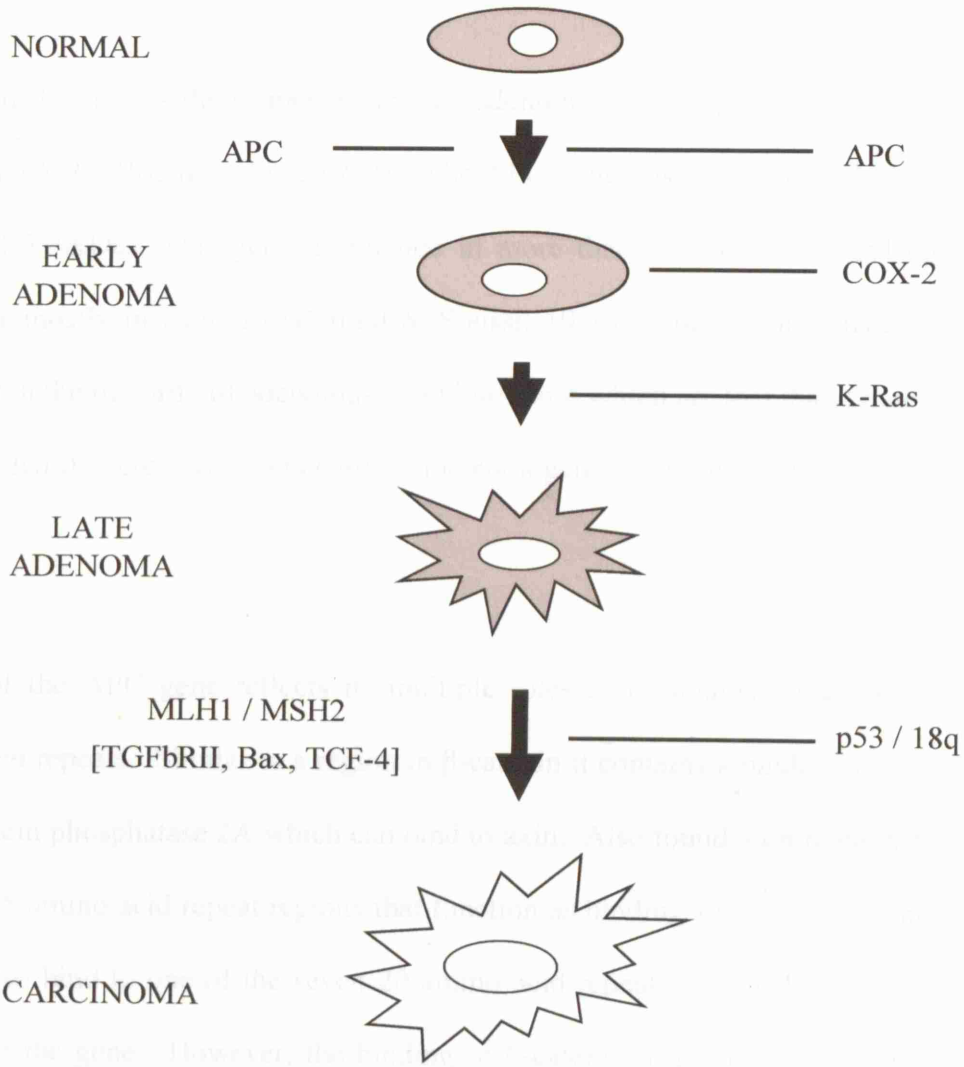


Figure 1.1
Schematic diagram of the pathogenesis of CRC, modified from Fearon and Vogelstein (1990). APC – Adenomatous Polyposis coli gene, COX-2 –Cyclooxygenase 2, K-Ras – Kirsten rat sarcoma 2 viral oncogene homologue, p53 – Protein 53, p18 - long arm chromosome 18, MLH1 – Mut L homologue mismatch repair gene, MSH2 – Mut S homologue mismatch repair gene, TGFβRII – Transforming growth factor beta receptor type two, Bax –BCL2-associated X protein, TCF-4 – Ternary complex factor 4.

In most CRCs the mutations occur in the partial loss of chromosomes 17p, 18q and 5q resulting in loss of heterozygosity with the possibility of mutated genes becoming dominant. A number of such genes thought to be important in CRC have now been identified (Cho & Vogelstein, 1992, Gayet *et al.*, 2001).

Chromosome 5q21 contains the sequences for the adenomatous polyposis coli tumour suppressor gene (APC; Bodmer *et al.*, 1987). The APC gene has 15 exons and codes for a protein of 310kDa. This gene is mutated in more than 75% of CRC, and the mutations occur mostly in exon 15 (Beroud & Soussi, 1996). These mutations have also been found in the majority of adenomas including those which are less than 5mm in size, indicating that this process occurs early in the pathogenesis of a tumour (Powell *et al.*, 1992).

The structure of the APC gene reflects its multiple roles and contains an armadillo region with seven repeats. Similar to a region in β-catenin it contains a binding site for the enzyme protein phosphatase 2A which can bind to axin. Also found within the APC gene are three 15 amino acid repeat regions that function as binding sites for β-catenin. β-catenin can also bind to one of the seven 20 amino acid repeat regions found in the central region of the gene. However, the binding of β-catenin at these sites can only occur after phosphorylation of glycogen synthetase kinase 3ß (GSK3β).

The APC protein has several roles in the maintenance of the colonic epithelium. APC protein predominates in the epithelial cells towards the tip of villi within the mucosa and stimulates epithelial cell differentiation and contributes to the control of the lifespan of these cells. APC also contributes to cell to cell adhesion, stability of the cell, cell

cycle regulation and apoptosis of cells but the majority of end results of APC function occurs via the Wingless Int 1 (WNT) signalling pathway.

The WNT signalling pathway is important in the development and maintenance of cells. After APC is phosphorylated by GSK3β it forms a complex consisting additionally of axin, GSK3β and β-catenin. This complex functions to destabilise β-catenin so it can be degraded by ubiquitin-mediated proteolysis. However if APC is mutated, β-catenin does not get degraded and so can accumulate in the cytoplasm and then the nucleus. Here, β-catenin binds to T cell factor (TCF) and lymphoid enhancer factor (LEF) to activate the main target genes cyclin D and c-myc, important genes in regulating progression of the cell cycle. As such, APC contributes to the control of the cell cycle by inhibiting progression into the S phase of the cell cycle via cyclin D and c-myc (Heinen *et al.*, 2002). A diagram of the WNT pathway is shown in Figure 1.2.

A separate function of APC is in cell to cell adhesion by competing with E-cadherin to bind β-catenin. E-cadherin is responsible for cell to cell adhesion in epithelial cells; in normal intestinal cells the epithelium is continually replaced by new cells, while old epithelial cells are shed into the lumen of the bowel at the tip of the villi, is where the highest concentration of APC protein is found. It is believed that APC may have a role in stimulating apoptosis of old epithelial cells prior to shedding (Fearnhead *et al.*, 2001).

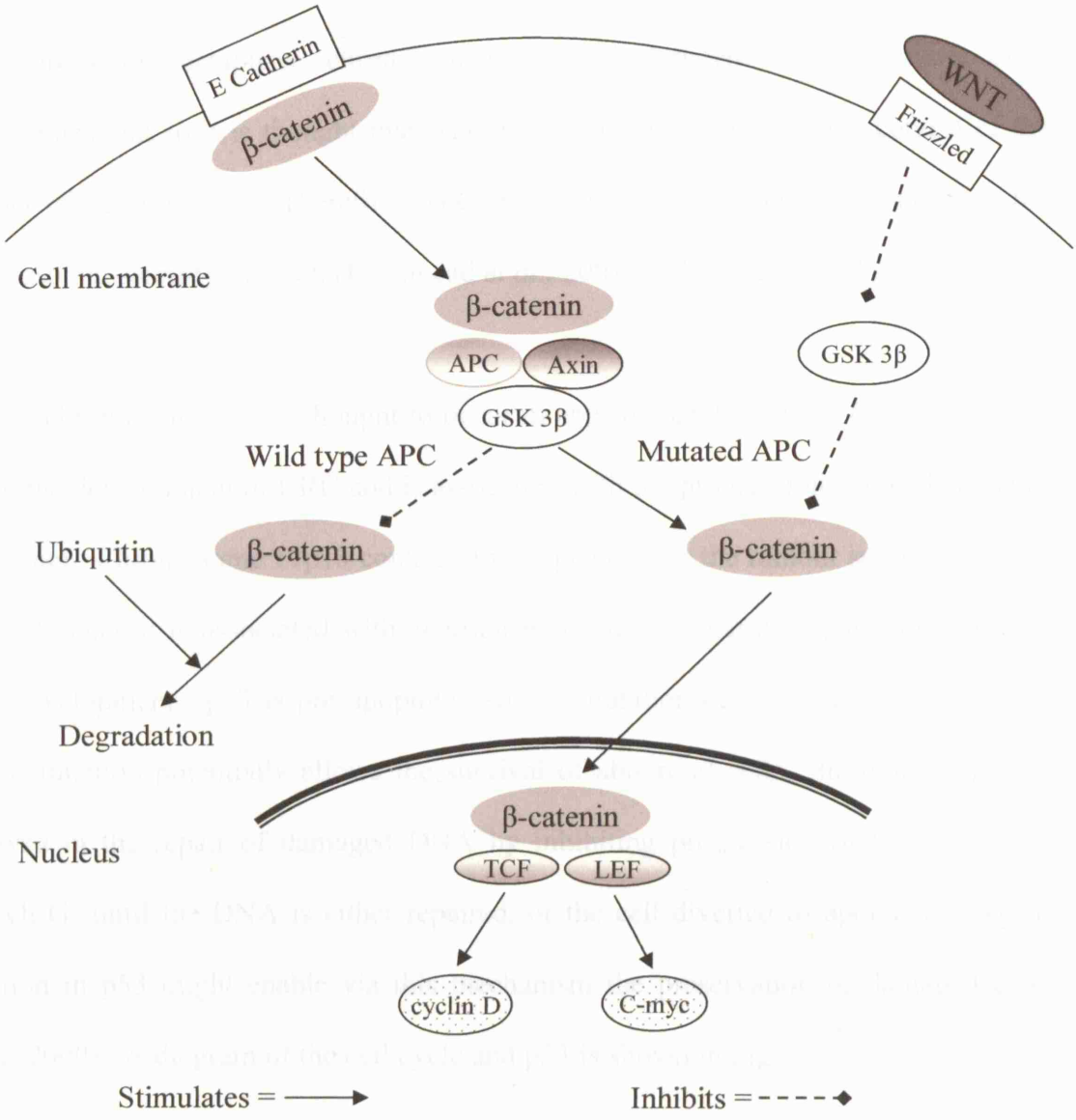


Figure 1.2
A schematic diagram of the Wingless Int 1 (WNT) pathway and β-catenin and E cadherin. Adapted from Hanahan & Weinberg (2000).

During mitosis the C terminal of APC binds to microtubules and the end binding protein (EB1) to maintain chromosomal stability by ensuring that the microtubules bind to kinetochores of metaphase chromosomes. Cells deficient in APC have extra centrosomes and so it is thought that APC has an additional role in the control of the number of centrosomes. Therefore APC function is an essential contribution to the chromosomal stability of a cell (Fearnhead *et al.*, 2001, Fodde *et al.*, 2001).

LOH at chromosome 17p is thought to occur in approximately 75% of CRC. It occurs late in the development of CRC and is associated with the progression from adenoma to carcinoma. Chromosome 17p13 contains the sequences for the tumour suppressor gene p53 and hence it is associated with aberrant p53 function that may play some role in CRC development. p53 is pro-apoptotic, so if a mutation occurs in p53 the effective loss of function potentially allows the survival of abnormal cells. In addition p53 is involved in the repair of damaged DNA by inhibiting progression of the cell cycle through G_1 until the DNA is either repaired, or the cell diverted to apoptosis. Again mutation in p53 might enable via this mechanism the preservation of damaged cells (King, 2000). A diagram of the cell cycle and p53 is shown in Figure 1.3.

LOH at 18q occurs in approximately 70% CRC and 50% of late adenomas (Fearon & Vogelstein, 1990). SMAD2 and SMAD4 comprise tumour suppressor genes found on chromosome 18q and which are components of the transforming growth factor-ß (TGF-ß) signalling pathway. TGF-ß functions to control cell proliferation, differentiation, motility, adhesion and apoptosis, and with its role in a specific cell depending on the type of cell. TGF-ß binds to TGF-ß type II receptor which becomes activated following

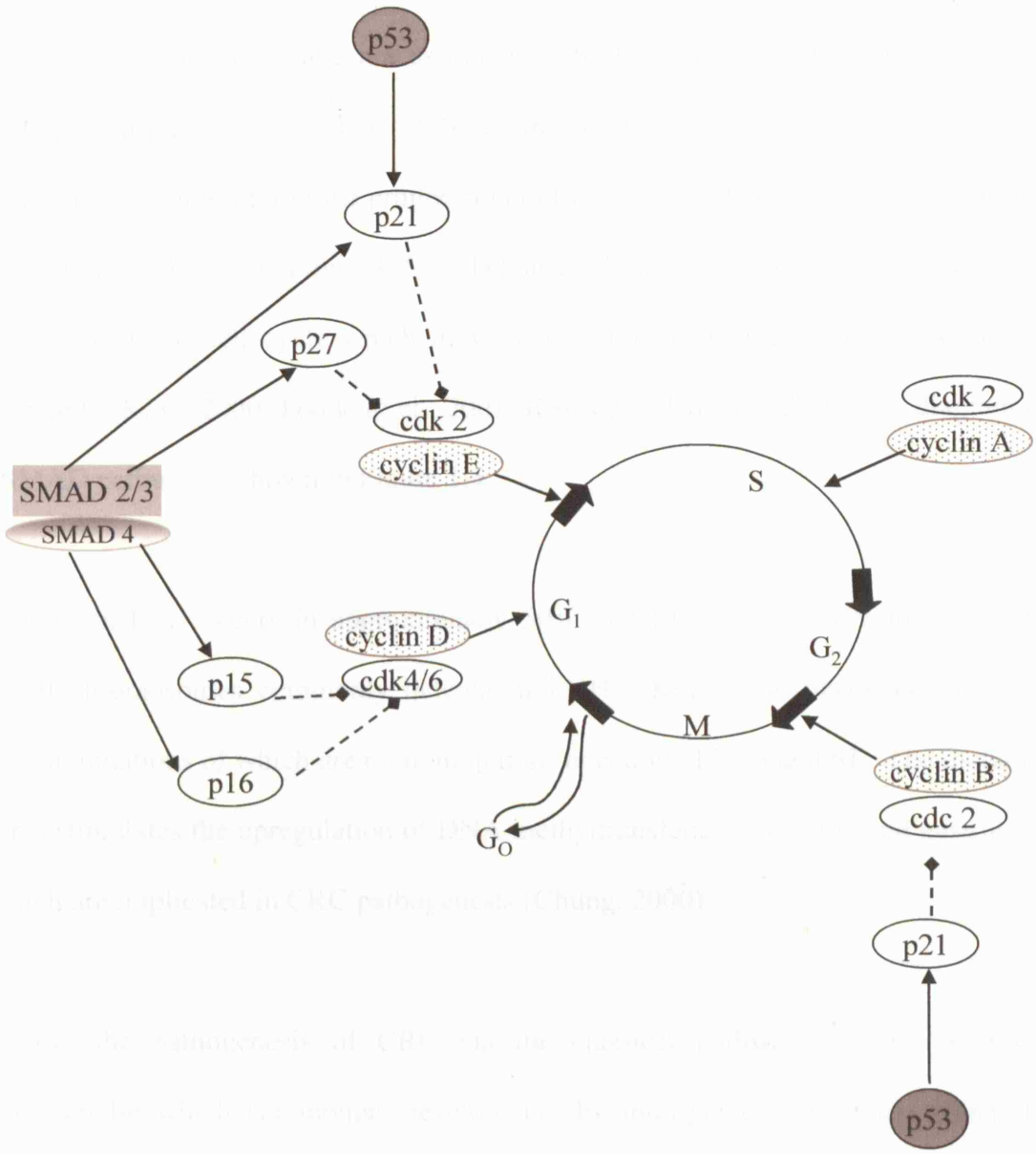


Figure 1.3
Modified diagram of the cell cycle and the action of p53 and SMAD. SMAD – Sma Mothers Against Decapentaplegic. Adapted from King (2002).

phosphorylation and then binds to SMAD2 and SMAD3. The resulting complex binds with SMAD4 and translocates to the nucleus where it activates a number of target genes. The target genes have not as yet been fully determined but p21, PAI 1 and JUNB have so far been identified. JUN is a transcription factor whilst p21 is a cyclin-dependent kinase inhibitor in the progression of the cell cycle from G_1 to S, and from G_2 to M phases. The alteration of SMAD2 and SMAD4 can allow upregulation of progression of the cell cycle which may in turn, potentiate the clonal expansion of cancer cells (King, 2000, Fodde *et* al., 2001, Rossant & Howard, 2002). A diagram of the SMAD pathway is shown in Figure 1.5.

Mutations in Kras occurs in approximately 50% of CRC, although not found on the parts of chromosomes commonly deleted in LOH. Kras is an oncogene the most common mutations of which are point mutations at codons 12, 13 and 61. The mutation of Kras stimulates the upregulation of DNA methyltransferase, cyclin D and gastrin, all of which are implicated in CRC pathogenesis (Chung, 2000).

Therefore the pathogenesis of CRC via the classical pathway fits the proposed mechanism by which carcinomas develop, i.e. by multiple events which affect the mechanisms involved in maintenance of the normal cell. There is however, an alternative pathway that has been described in the development of CRC.

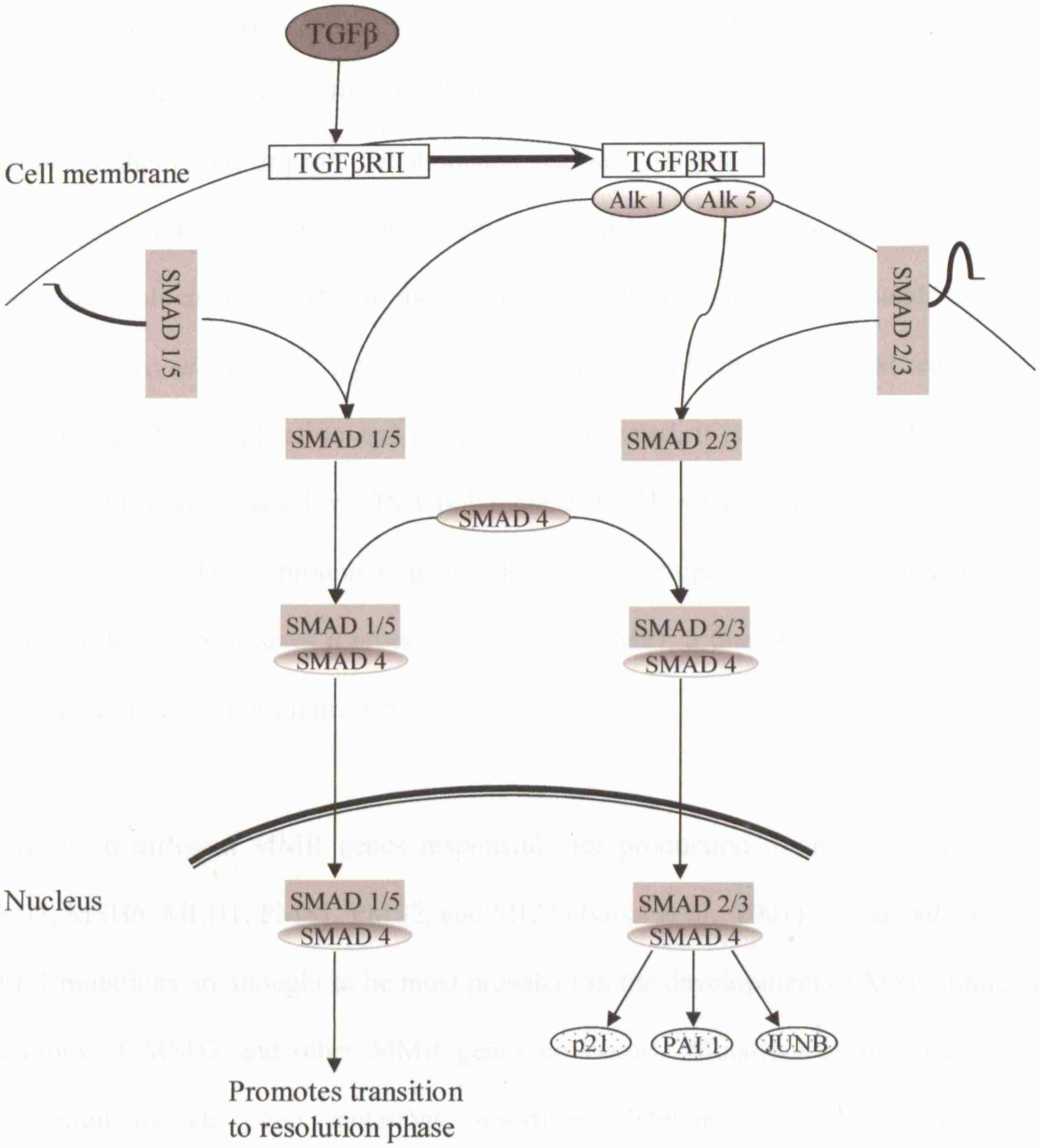


Figure 1.4
Schematic diagram of the SMAD pathway. Adapted from Lutz & Knaus (2002).

1.2.3 Alternative pathway of carcinogenesis

The alternative pathway of CRC development is seen in approximately 15% of sporadic CRC and is due to microsatellite instability (MSI). The problem arises when there are repeats in either exons or promoter/attenuator regions – so gene activity and/or function becomes dysregulated. Genes which contain simple repeats of poly A or CA are the most susceptible to errors. Due to the greater susceptibility to errors, a separate class of proteins known as mismatch repair (MMR) proteins 'proof read' the repeat regions for errors during DNA replication and if an error is detected, it is excised and the correct DNA is then re-synthesised by DNA polymerase A. However if mutation occurs in a MMR gene, the altered protein is unable to function properly allowing mutations to remain in these repeat areas a process described as MSI; a diagram of the function of MMR genes is shown in Figure 1.6.

There are 6 different MMR genes responsible for production of the MMR proteins MSH2, MSH6, MLH1, PMS1, PMS2, and MLH3 (Goel *et al.*, 2001). In sporadic CRC, MLH1 mutations are thought to be most prevalent in the development of MSI, although mutations of MSH2 and other MMR genes do occur. Mutations resulting in gene inactivation include point mutations, insertions, deletions, frameshifts and even chromosomal deletions. In addition to mutation, it is thought that methylation of the CpG islands in the MLH1 promoter region is also responsible for the silencing of the gene and so loss of MMR function (Goel *et al.*, 2001, Jass, 2002).

Sporadic tumours with MSI differ in genetic characteristics to non-MSI tumours. For example, there is decreased APC mutation with a corresponding decrease in LOH of chromosome 5q. In addition, there is a reduced p53 and Kras mutations, LOH of 17p

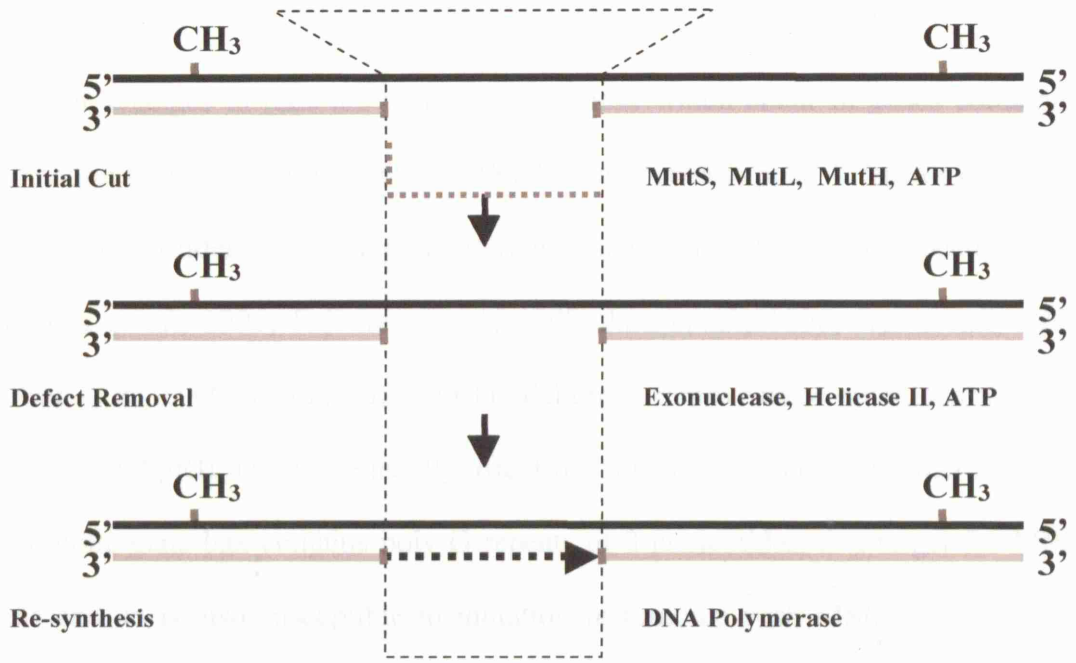


Figure 1.5
Schematic diagram of the role of the Mismatch Repair (MMR) proteins. MMR proteins survey a DNA sequence for errors, if an error is detected it is excised and the correct sequence is synthesised.

and 18q. The WNT signalling pathway is thought to be intact in MSI as there is no increase in expression of β-catenin, or indeed mutation of β-catenin (Jass *et al.*, 1999, Gayet *et al.*, 2001). Also in contrast to tumours with chromosomal instability (CIN) in tumours with MSI the DNA of cells is diploid. Tumours with MSI do not seem to arise from pre-existing adenomas but MSI possibly from serrated or hyperplastic adenomas (Jass *et al.*, 2002).

The genetic changes in tumours with MSI are those which occur in genes containing microsatellite regions. For example, TGFβRII gene contains poly A repeats consisting of 10 mononucleotides which are prone to mutation and indeed commonly occur in MSI (Markowitz *et al.*, 1995, Fujiwara *et al.*, 1998, Jass *et al.*, 1998, Jass *et al.*, 1999, Barnetson *et al.*, 2000, Young *et al.*, 2001). Failure to remove these mutations alters the function of TGFβRII and consequently affect the function of TGFβ. Furthermore, the anti apoptotic gene bax contains poly G repeats of 8 nucleotides, is a target for MMR proteins and so is also susceptible to mutation in tumours with MSI. Although bax mutations do not occur as frequently as in TGFβRII this may be due to the smaller length of the repeat region (Fujiwara *et al.*, 1998, Bacon *et al.*, 2001). Mutations have also been detected in INFRA, MSH3 and MSH6 genes in MSI tumours but they are not as common (Barnetson *et al.*, 2000).

Tumours with MSI have clear and altered pathological characteristics. MSI tumours tend to be poorly differentiated and mucinous adenocarcinomas, with heavy lymphocyte infiltration (Jass *et al.*, 1998, Chao *et al.*, 2000, Young *et al.*, 2001).

1.2.4 Microsatellite instability and DNA Mismatch Repair

'MSI is caused by a failure of the DNA MMR system to repair errors that occur during replication of DNA and is characterised by the accelerated accumulation of single nucleotide mutations and alterations in the length of simple, repetitive microsatellite sequences that occur ubiquitously through out the genome' (Boland *et al.*, 1998). The National Cancer Institute, 1998 determined the mirosatellites that can be used as markers to identify tumours with MSI. Microsatellites (markers) that contain mutations indicates that the MMR proteins are mutated and have not corrected the errors, and the tumour is described as having MSI.

A reference panel of five microsatellites have been suggested to be used to identify tumours with MSI:

Marker	Repeating unit	GenBank accession no.
BAT 25	Mononucleotide	9834508
BAT 26	Mononucleotide	9834505
D5S346	Dinucleotide	181171
D2S123	Dinucleotide	187953
D17S250	Dinucleotide	177030

Tumours are classified according to how 'unstable' they are depending on the number of markers which contain mutations:

MSI classification	No. markers unstable
Stable (MSS)	0
Low (MSI-L)	< 30-40%
High (MSI-H)	≥ 30-40%

Mononucleotide markers are thought to be more sensitive at detecting high-level MSI (MSI-H) tumours while dinucleotide markers are thought to be more sensitive for tumours with low-level MSI (MSI-L), which also appears to be true even if there is more than one dinucleotide marker which is unstable which normally would be classified as MSI-H. Jass and co-workers (2002) have expressed concern that the inclusion of tumours which have more than one dinucleotide repeat marker and no mononucleotide markers that are unstable, may not in fact be true MSI-H tumours. They suggested that these tumours are really MSI-L tumours as these tumours have different phenotypes to true MSI-H tumours and so their inclusion within the MSI-H group contributes to the difficulty classifying these tumours as a distinct pathological group.

Compared to MSI-L, MSI-H tumours have a low frequency of APC mutation and fewer mutations of β catenin and Kras (Jass *et al.*, 1999, Jass *et al.*, 2002, Whitehall *et al.*, 2002). Furthermore MSI-H tumours demonstrate mutation of MLH1 and MSH2 genes whereas there appears to be little or no loss of expression of these genes in MSI-L. This may help to explain why MSI-L tumours demonstrate a different phenotype to MSI-H.

There is controversy regarding the differences between MSI-L and MSS cancers. MSI-L tumours have LOH of chromosome 1p and a higher frequency of Kras mutations

when compared to MSS and a higher rate of cellular turnover. However frequencies of p53 and APC mutations in MSI-L and MSS tumours are similar (Konishi *et al.*, 1996), and do not show definite differences in clinicopathological features (Tomlinson *et al.*, 2002).

MSI-L is prevalent in early CRC which also show increased LOH of 1p32 and 8p12-22 when compared to MSS, whereas advanced CRC, MSI-L tumours also have LOH of 2p16, 7q31 and 17q11. Therefore the biological basis of MSI-L is not clearly understood and the different phenotype may be the result of differences in LOH compared to MSI-H or MSS tumours (Kambara *et al.*, 2001). It has also been suggested that the differences in MSI-L might be due to methylation of the promoter region of the DNA repair gene O^6methylguanine DNA methyltransferase (MGMT) although this has only been demonstrated in a subset of MSI-L tumours (Whitehall *et al.*, 2001).

The different genetic alterations which occur in MSI-H, MSI-L and MSS tumours might account for their different phenotypes. MSI-H tumours are predominantly found in the right colon (Chao *et al.*, 2000, Gryfe *et al.*, 2001), in female (Ward *et al.*, 2001) and elderly patients (>70 years; Chao *et al.*, 2000). They have lower incidence of lymphatic and distant spread and a better prognosis when stage matched with MSS tumours (Gryfe *et al.*, 2001, Wright *et al.*, 2000). Furthermore MSI-H tumours have been shown to have an improved response to chemotherapy (Elsaleh *et al.*, 2000, Elsaleh *et al.*, 2001). Whereas tumours with the MSI-L phenotype are more common in the left colon and rectum (Jass *et al.*, 1999), and are more aggressive with an increased risk of metastatic spread (Jass *et al.*, 1999), have a higher recurrence rate and a poorer prognosis (Kambara *et al.*, 2001).

1.2.5 Micosatellite and chromosomal stable pathway

A further group of CRC have recently been identified which are both microsatellite and chromosomal stable (MACS) (Georgiades *et al.*, 1999, Chan *et al.*, 2001). These tumours have near diploid DNA content, have few chromosomal imbalances and have microsatellite stable DNA.

MACS CRC are most commonly found in patients younger than 45 years old, in the distal colon and rectum and are moderately differentiated. When MACS tumours are staged matched with MSI-H tumours they have a poorer prognosis (Chan *et al.*, 2001). Chan *et al.* (2001) hypothesised that these tumours gain the malignant phenotype via a third pathway or MACS tumours will eventually develop into a tumour with CIN.

Prognosis in CRC is related to the spread of the tumour through the bowel wall and its' lymphovascular spread, mediated through the vascular endothelial growth factor (VEGF) family of cytokines. Vascular endothelial growth factor A ($VEGF_A$) has been suggested to be one protein which may in part explain these differences.

1.3 Vascular Endothelial Growth Factor A

1.3.1 Angiogenesis

Angiogenesis is the process by which new blood vessels are produced during normal tissue growth, and also occurs in certain pathological conditions that involves the expansion of pre-existing blood vessels by sprouting of the endothelium with infrequent stabilisation of the newly formed vessels. Whereas, vasculogenesis involves the formation of blood vessels by endothelial progenitors. Both angiogenesis and vasculogenesis occur in the adult as well as the embryo (Figure 1.6).

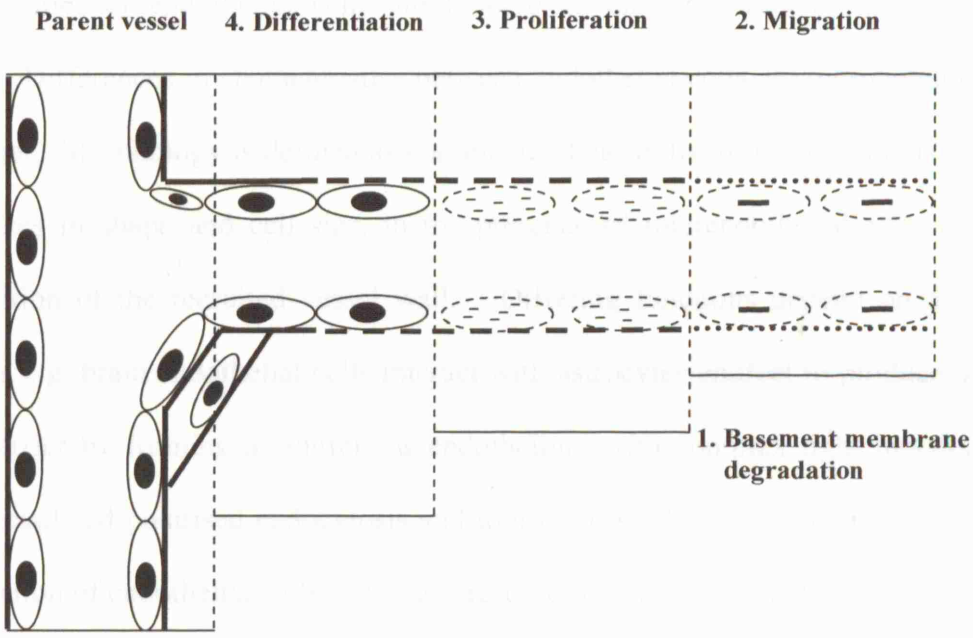


Figure 1.6
Schematic of steps in angiogenesis modified from Kumar *et al.* (1997). The parent mature vessel is on the left. 1, Basement membrane and ECM matrix degradation. 2, Endothelial migration. 3, Endothelial proliferation. 4, Organisation and maturation.

Endothelial Progenitors

The endothelial cells in the embryo differentiate from angioblasts and in adults from endothelial progenitor cells, mesangioblasts, and multipotent adult progenitor cells in the bone marrow. Endothelial progenitor cells can also stimulate angiogenesis by releasing angiogenic growth factors, and are controlled by signals which stimulate their recruitment or differentiation both as the embryo and the adult (Carmeliet *et al.*, 2003).

Endothelial Differentiation

Endothelial progenitors differentiate into mature endothelial cells which may have different cellular properties depending on vessel type, the organ and age. The particular characteristics of endothelial cells are tailored to the environment where they are located. Differences in characteristics between endothelial cells are morphological and functional. Morphology is defined as continuous, fenestrated or discontinuous and also differences in shape and cell size, in the presence of interendothelial adhesions and composition of the recruited vessel wall. Differing functions depend on the organ location, e.g. brain endothelial cells interact with astrocytes endfeet to produce a blood brain barrier by forming a continuous endothelium with complex tight junctions and highly regulated polarised endocytosis and transcytosis. These differences in structure and function of endothelial cells occur as a result of different molecular signals (Cleaver & Melton, 2003).

Molecular signalling of endothelial cells

The formation of a mature blood vessel involves the maturation of endothelial cells, mural cells and matrix, a process which occurs in stages; formation, stabilisation, branching, remodelling, pruning and specialisation.

Formation of immature blood vessels

In the embryo vessels form by both vasculogenesis and angiogenesis, by a process thought to be initiated by the molecule vascular endothelial growth factor ($VEGF_A$). $VEGF_A$ is also involved in instigating a chain of molecular and cellular events which results in the formation of mature blood vessels. CD31, CD34 and VEGF-2 positive receptor angioblasts form a vascular plexus which results in the formation of the distal aorta, the cardinal vein and the embryonic stems of the arteries and veins (Carmeliet, 2003).

Hypoxia is thought to instigate sprouting angiogenesis which increases the size of the vascular bed. Initially cells are oxygenated by simple diffusion of oxygen but as the tissue grows beyond the limit of oxygen diffusion, the cells become hypoxic which upregulates the expression of a number of genes involved in vessel formation and maturation. Angiogenesis involves several steps; retraction of pericytes, degradation of extracellular matrix surrounding the capillaries, migration and proliferation of endothelial cells, formation of capillary tubes by endothelial cells and formation of anastomosis and establishment of blood flow between sprouting vessels and existing vessels (Carmeliet & Jain, 2000).

Hypoxia also stimulates the up-regulation of nitric oxide synthetase (NOS) responsible for the production of nitric oxide (NO). NO stimulates existing vessel dilatation and $VEGF_A$, also upregulated by hypoxia, and increases vessel wall permeability. The basement membrane and extracellular matrix is broken down by activation of proteases, such as matrix metalloproteinase MMP-2, MMP-3 and MMP-9. Plasma proteins then leak from the existing blood vessels and act as a provisional matrix. $VEGF_A$ stimulates

endothelial cells to migrate through interactions between integrins and the matrix and along with angiopoietin (Ang) 2, facilitates sprouting and the formation of vascular loops and networks (Jain, 2003).

Stabilisation of blood vessels

Immature blood vessels become stabilised following the recruitment of mural cells and the generation of extracellular matrix; a process that involves several different signalling pathways.

Platelet derived growth factor (PDGF) and its receptor PDGF-β, are both important in stabilising immature blood vessels by recruiting PDGFR-β positive mesenchymal progenitors (Jain, 2003).

Another pathway important in the stabilisation of blood vessels is via the ligands Ang 1 and Ang 2, mainly produced by mural cells and endothelial cells, and their Tie receptors, Tie 1 and Tie 2. Ang 1 stabilises cells and facilitates communication between endothelial cells and mural cells resulting in vessels that are leak resistant (Suri *et al.*, 1998). Ang 2 has two different roles, both facilitating vessel sprouting in the presence of $VEGF_A$ and in the absence of $VEGF_A$ causing endothelial cell death and blood vessel regression (Lougha & Sato, 2001a).

The cytokine TGFβ (along with its other functions) stimulates extracellular matrix production and induces differentiation of mesenchymal cells to mural cells, both of which promote vessel maturation. However TGFβ at low levels contributes to the

upregulation of angiogenic factors and proteinases required for new blood vessel growth (Rossant & Howard, 2002).

Branching, remodelling and pruning of blood vessels

This organisation results in the production of the optimal vessel network. Signalling pathways which regulate branching in the nervous system are involved in the regulation of proliferation, survival, migration and differentiation of endothelial and mural cells. These pathways also control the various basement membrane and extracellular matrix components (Mukoayama *et al.*, 2002, Neufeld *et al.*, 2002). The extracellular matrix serves as a 'store' for growth factors and pro-enzymes involved in blood vessel development. Degradation of the basement membrane and the extracellular matrix is controlled by proteases whose inhibitors influence endothelial cell and mural cell migration. Proteases also release both pro-angiogenic factors such as $VEGF_A$ and basic fibroblast growth factor (bFGF), and anti-angiogenic factors such as plasma proteins or matrix molecules. It is the levels of pro-and anti-angiogenic factors that affect branching of vessels by regulating proliferation and apoptosis of endothelial cells and mural cells (Kalluri, 2003).

Vessel specialisation

Specialisation involves the formation of veins and arteries, different endothelial junctions and endothelial cell differentiation to form organ-specific capillary structures (Jain, 2003). The development of venous and arterial vessels is determined early in the development of the vascular system (Patan, 2000).

The Notch pathway is thought to be important in the development of arteries as opposed to veins. The Notch gene was first identified in *Drosophila* and encodes for a 300kDa single-pass transmembrane protein (Artavanis–Tsakonas *et al.*, 1999). The four known Notch receptors (1-4) interact with the five ligands Jagged 1, Jagged 2, Delta like(Dll) 1,3, and 4 all of which are expressed in and around developing vasculature (Kojika & Griffin, 2001, Nye & Kopan, 1995). While Notch 1, Notch 4, Delta 4, Jagged 1 and Jagged 4 are expressed in arterial endothelium, they are at only low levels (or not at all) in the venous epithelium. Jagged 1 is expressed in both endothelial and smooth muscle cells of arterial vessels, Notch 4 and Delta 4 are only in endothelium. The Notch signalling pathway may therefore be responsible for the suppression of venous vessel formation (Uyttendaele *et al.*, 1996, Shirayoshi *et al.*, 1997, Shutter *et al.*, 2000).

Ephrin are membrane bound ligands binding to Eph receptors (Xu & Wilkinson, 1997, Flanagan & Vanderhaeghen, 1998). Ephrin B2 is expressed in endothelial cells of arteries whilst ephrin B4 is expressed in venous endothelial cells (Wang *et al.*, 1998). This differential expression of ephrin is found early in the development of blood vessels and may indicate that endothelial cells are genetically determined to become venous or arterial. It is thought that bi-directional signalling between mesenchymal cells and endothelial cells may help to separate venous and arterial cells and correct the development of arteries and veins and possibly contribute to vessel branching (Patan, 2000).

Physiological angiogenesis

The process of angiogenesis and maturation of new blood vessels is also involved in wound healing, menstrual cycling and ocular maturation. Hypoxia, low pH, altered

hydrostatic and shear stress are changes in local metabolic and mechanical environment which stimulate physiological angiogenesis (Topper & Gimbrone, 1999, Semenza, 2001, Xu *et al.*, 2002).

Wound healing provides a good example of physiological angiogenesis; in the early stages of wound healing a large number of immature vessels form. Later, some blood vessels become pruned and the remaining vessels mature (Zawiciki *et al.*, 1981). Molecular signals that change during this process are, for example, $VEGF_A$ and Ang 2 expression which increase initially and then decrease to a baseline level after a stable vascular network is formed, whereas Ang 1 expression transiently decreases after wound formation and then decreases further after blood vessel maturation (Bloch *et al.*, 2000).

Angiogenesis and cancer

A tumour starts as a cluster of cells and to develop and progress new blood vessel growth is required. Gimbrone *et al.* (1972) demonstrated that when tumour cells were placed near the iris of the eye they attracted blood vessels and subsequently the number of tumour cells grew exponentially. However, when the tumour cells were placed in the anterior chamber of the eye where they could not recruit blood vessels there was no increase in tumour cell number.

Tumours have the capability to instigate angiogenesis as part of their phenotype. The process(es) underlying this are known as the 'angiogenic switch' and occur early, or during the pre-neoplastic stage. Induction of the angiogenic switch depends on the balance between pro- and anti-angiogenic factors (Carmeliet & Jain, 2000).

Tyrosine kinase receptor ligands are known to be potent inducers of angiogenesis, especially true when both $VEGF_A$ and bFGF molecules work synergistically. Inhibitors of angiogenesis for example thrombospondin-1 (TSP-1) and statins such as angiostatin and endostatin have important effects on the 'switching-on' of the angiogenic switch. TSP-1 is controlled by the wild type p53 tumour suppressor protein. Therefore tumours with p53 mutations and compromised p53 function are rendered unable to control TSP-1, which results in activation of the angiogenic switch (Hanahan & Folkman, 1996).

Pathological angiogenesis has different characteristics to those of physiological angiogenesis. Tumour blood vessels are irregularly shaped, tortuous, dilated and can have dead ends, and have a constant capability to grow new blood vessels. These new vessels have a chaotic pattern and demonstrate characteristics of both arteries and capillaries, are leaky and can be haemorrhagic, which may be due to overproduction of $VEGF_A$. The blood flow within tumour vessels instead of being uniform is slow and can oscillate causing dysfunctional capillaries (Bergers & Benjamin, 2003).

Tumour blood vessels form by the materialisation of endothelial precursor cells from vessel walls or bone marrow to the site of growing blood vessels. Their formation is instigated by pro-angiogenic factors stimulating pre-existing blood vessels to vasodilate and increase permeability (Yancopoulos *et al.*, 2000). This leads to extravasation of plasma proteins which lay down an extracellular matrix into which activated endothelial cells can migrate. The next stage is the degradation of vascular basement membrane and extracellular matrix allowing further endothelial cells to migrate into the perivascular space. The endothelial cells then multiply to form a column of cells. Normally endothelial cells would then organise and adhere to each other to form an

organised lumen. However this does not occur in tumour blood vessels and the end result is abnormal vessel diameters and altered responses to angiogenic factors (Baish & Jain, 2000). Furthermore tumours can independently produce angiogenic factors such as $VEGF_A$, FGF and placental growth factor (PlGF) which further stimulate angiogenesis and so the cycle self perpetuates (Jain *et al.*, 1996, Huang *et al.*, 1997, Eliceiri & Cherresh, 1999).

1.3.2 Molecular structure

$VEGF_A$ is a 46kDa protein of the VEGF family of proteins, derived from the gene located at chromosone 6p21.3. The other members of the VEGF family share homology and interact with each others' cell surface receptors (Erikson & Alitalo, 2000), and include $VEGF_B$, $_C$ and $_D$. $VEGF_B$ is thought to have a role in coronary vascularisation and growth as mice lacking $VEGF_B$ look normal and are fertile, but their hearts are smaller (Bellomo *et al.*, 2000). $VEGF_C$ has been found to have a role in lymphangiogenesis and is discussed in Section 1.1.4. $VEGF_D$ has a role along side $VEGF_C$, in lymphangiogenesis. The rest of this section will discuss the structure and function of $VEGF_A$.

$VEGF_A$ consists of eight exons with alternative splicing of single mRNA generating different isoforms. Exon one and exon two, four residues, code for the signal peptide which is essential for protein secretion (Leung *et al.*, 1989). The four main isoforms are; $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, which can be divided into soluble ($VEGF_{121}$ and $VEGF_{165}$) and insoluble isoforms ($VEGF_{189}$, and $VEGF_{206}$). There are less frequent slice variants which include $VEGF_{145}$ and $VEGF_{183}$ (Figure 1.7).

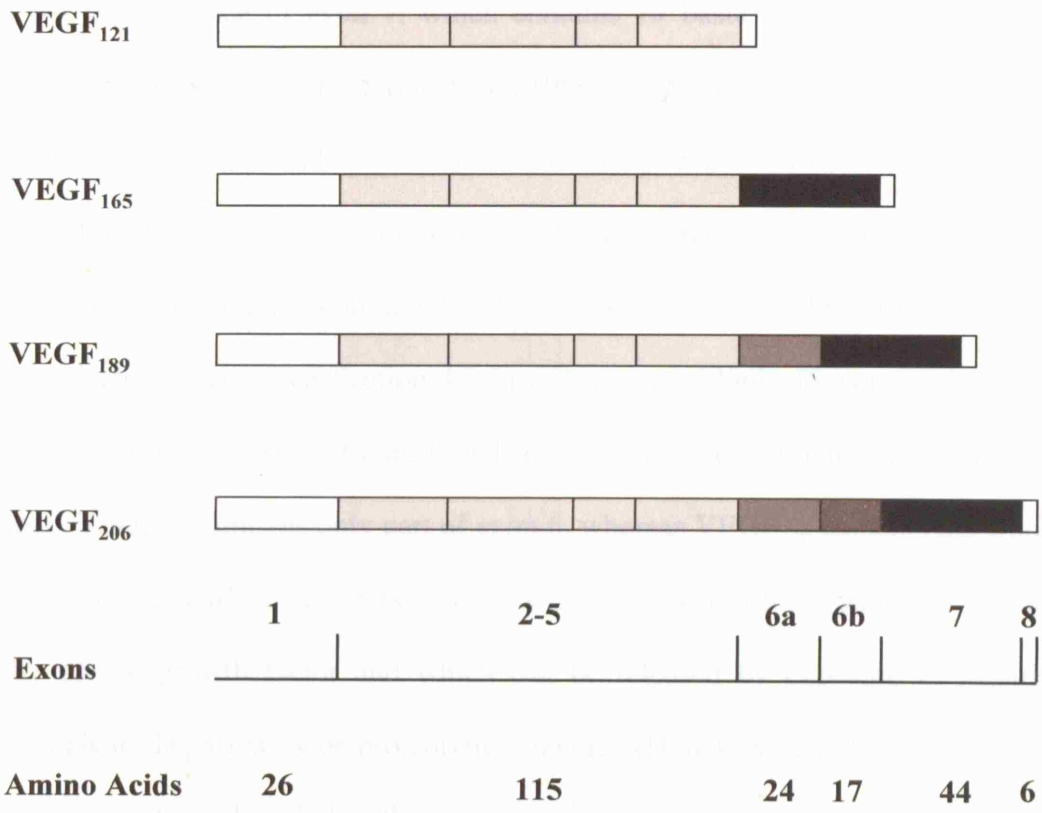


Figure 1.7
$VEGF_A$ isoforms 121, 165, 189 and 206, plus the exons and amino acids which make up the full length mRNA. Modified from Robinson & Stringer, (2001).

The soluble isoforms are freely diffusable molecules, secreted by cells and this is especially true for $VEGF_{121}$. $VEGF_{121}$ lacks exons 6 and 7 and is a weakly acidic protein that does not bind to heparin (Houck *et al.*, 1992). Whereas $VEGF_{165}$ lacks exon 6 but the presence of exon 7, which contains 15 basic amino acids, gives the protein its basic properties (Ferrara & Henzel, 1989, Gospodarowicz *et al.*, 1989, Plouet *et al.*, 1989). $VEGF_{165}$ binds to heparin which may explain why a proportion of $VEGF_{165}$ is found within cells and in the extracellular matrix (Houck *et al.*, 1992). The soluble isoforms stimulate endothelial cell mitogenesis by binding to the VEGF receptors (discussed later, see Section 1.3.3) (Keyt *et al.*,1996b, Plouet *et al.*, 1997). The insoluble isoforms (189, 206) are found solely in the extracellular matrix and bind to heparin. $VEGF_{189}$ contains only part of exon 6, whereas $VEGF_{206}$ contains the whole of exon 6 (Houck *et al.*, 1992, Park *et al.*, 1993). These insoluble isoforms provide reservoirs of the growth factor and which can be released by exposure to heparin, heparin sulphate, heparinases or proteolytic enzymes (Houck *et al.*, 1992). They are unable to stimulate endothelial cell mitogenesis as protein folding blocks receptor-binding regions (Houck *et al.*, 1991).

1.3.3 $VEGF_A$ receptor subtypes

$VEGF_A$ receptors are a family of tyrosine kinase receptors; VEGF-R1 (Flt-1), VEGF-R2 (KDR/Flk-1) and VEGF-R3 (Flt-4). Flt-1 has the strongest binding capacity for $VEGF_A$ and binds to three acidic residues locked in exon three of the $VEGF_A$ gene. In contrast, KDR/Flk-1 binds to $VEGF_A$ via three basic residues from exon four of $VEGF_A$ (Keyt *et al.*, 1998a). Flk-4 has barely any binding potential and is discussed in Section 1.4. A schematic of the ligands, receptors and resulting action is shown in Figure 1.8.

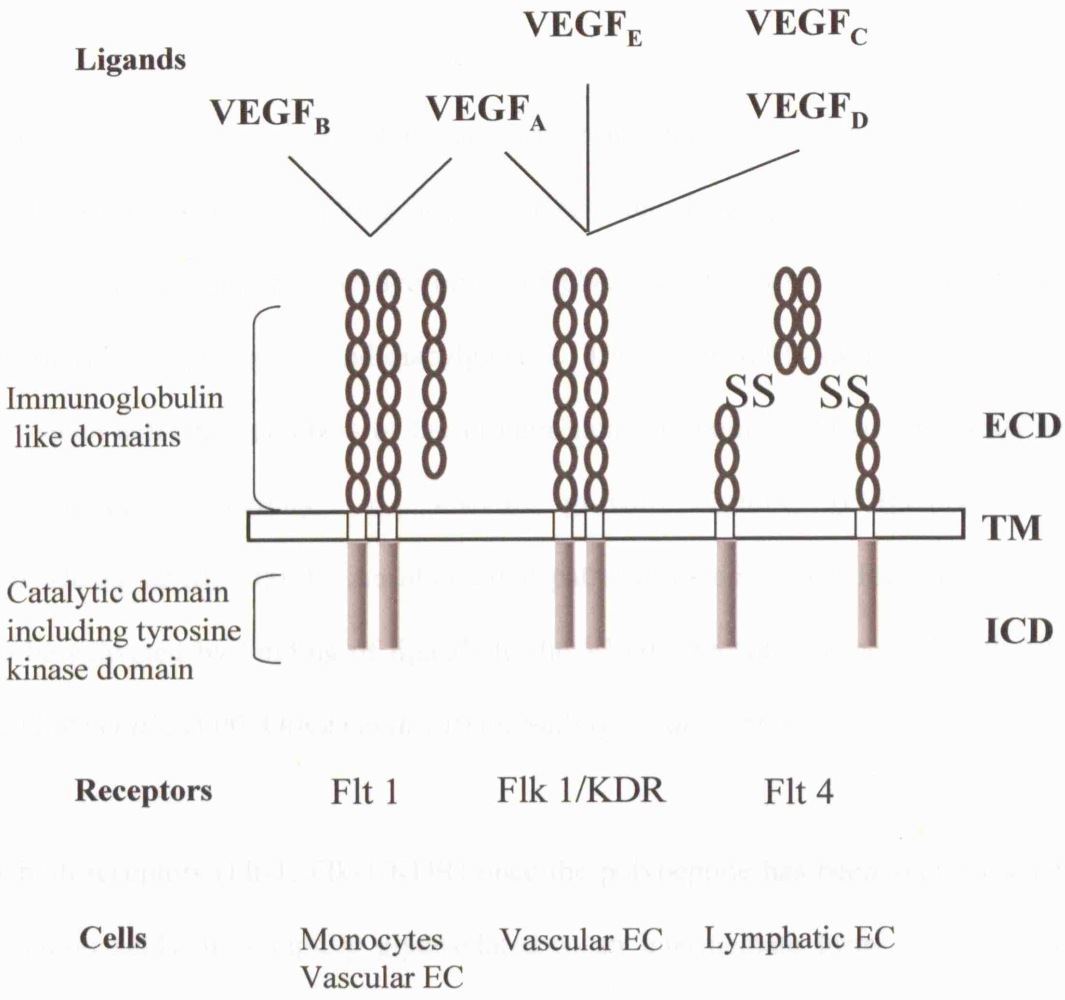


Figure 1.8
Diagram of VEGF receptors and ligands. Modified from Zachary (2001).

The Flt-1 and Flk-1/KDR receptors have seven immunoglobulin-like domains in their extracellular domain. There is a single transmembrane region and tyrosine kinase sequence which is interrupted by a kinase insert domain (Shibuya *et al.*, 1990, Terman *et al.*, 1992).

Binding of VEGF to its' receptors causes receptor dimerization and activation of the tyrosine kinase activity resulting in phosphorylation of phospholipase C (PLC). PLC then acts as a catalyst for the hydrolysis of phosphatidyl inositol 3,4 bisphosphate to inositol 1,4,5 trisphosphate and diacylglcerol. The end result is stimulation of calcium flux which activates members of the protein kinase C family which is responsible for the mitogenic activity of several growth factors (Carpenter & Ji, 1999, Huang *et al.*, 2001). An alternative pathway mediated via Ras-MEK-Erk, can also be activated by binding of ligands to the $VEGF_A$ receptors especially Flk-1/KDR (Veikkola *et al.*, 2000, Ortega *et al.*, 1999, Shibuya *et al.*, 1999).

For both receptors (Flt-1, Flk-1/KDR) once the polypeptide has been synthesised from the amino acids, it is rapidly glycosylated to an intermediate form which is further glycosylated and expressed on the cell surface as a mature protein of 180-185 kDa for Flt-1 (Shibuya *et al.*, 1990), and 200-230 kDa for Flk-1/KDR (Terman *et al.*, 1992). The mature form is then phosphorylated on ligation with $VEGF_A$. The immunoglobulin-like (Ig) extracellular domains of the receptors are crucial for binding with $VEGF_A$ (Hiratsuka *et al.*, 1998); the first to the third Ig domain is essential for $VEGF_A$ binding, with direct binding occuring at the 2nd Ig domain (Davis-Smyth *et al.*, 1996, Davis-Smyth *et al.*, 1998). The intracellular region of the receptor has a tyrosine kinase domain and a carboxyl terminal region downstream of the kinase domain.

Both Flt-1 and Flk-1/KDR are expressed in endothelial cells and signal for cell proliferation and vascular permeability. Flk-1/KDR has been demonstrated to stimulate a greater biological response than Flt-1 (Shibuya, 2001). Mice embryos deficient of Flk-1/KDR die *in utero* due to lack of yolk blood island formation and vasculogenesis, indicating a role for Flk-1/KDR in vasculogenesis and blood island formation (Shalaby *et al.*, 1995, Millauer *et al.*, 1993). In contrast Flt-1 may have a role in endothelial progenitor cell adhesion and the interaction with the extracellular matrix (Fong *et al.*, 1995). Monocytes and macrophages express significant levels of Flt-1 compared to Flk-1/KDR which may indicate a greater role in $VEGF_A$-dependent functions of monocytes and macrophages as demonstrated by $VEGF_A$-stimulated migration of human peripheral blood monocytes even in the presence of antibodies to Flk-1/KDR (Barleon *et al.*, 1996).

1.3.4 Regulation of $VEGF_A$ expression

There are several factors involved in the control of $VEGF_A$ gene transcription. In the embryo control of $VEGF_A$ gene transcription is essential as damage to just one allele can lead to death or severe vascular deformities (Carmeliet *et al.*, 1996, Ferrara *et al.*, 1996). Furthermore, the development of an embryo is subject to more subtle changes in levels of $VEGF_A$ isoforms (Carmeliet *et al.*, 1999, Miquerol *et al.*, 2000). In adults this constant control is not required except during physiological angiogenesis. This is demonstrated in diabetic retinopathy, when damage and loss of healthy blood vessels results in retinal hypoxia which leads to induction of $VEGF_A$ and an abnormal angiogenic response culminating in leaky haemorrhagic vessels (Adamis *et al.*, 1994, Aiello *et al.*, 1994). Therefore, appropriate induction of $VEGF_A$ without the complete pathway of angiogenesis results in the formation of leaky and immature vessels.

Several factors have been identified in the control of $VEGF_A$ expression; cytokines, hypoxia, p53, oestrogen, thyroid stimulating hormone, nitric oxide, and tumour promoters.

Cytokines may not have a direct role in instigating angiogenesis but they can have an indirect effect by altering the levels of $VEGF_A$ expression. Cytokines known to potentiate $VEGF_A$ production include FGF-4 (Deroanne *et al.*, 1997), PDGF (Finkenzeller *et al.*, 1997), TNF-α (Ryuto *et al.*, 1996), TNF-β (Pertovaara *et al.*, 1994), KGF (Frank *et al.*, 1995), IGF-I (Goad *et al.*, 1996), IL-1β (Li *et al.*, 1995) and IL-6 (Cohen *et al.*, 1996). Conversely production of $VEGF_A$ can be inhibited by IL-10 and IL-13 (Matsumoto *et al.*, 1997).

A key molecule implicated in the action of $VEGF_A$ is nitric oxide (NO) and which can upregulate $VEGF_A$ and enhances both blood vessel permeability effects of $VEGF_A$ and $VEGF_A$-stimulated vasodilation (Tuder *et al.*, 1995, Chin *et al.*, 1997, Murohara *et al.*, 1998).

Hypoxia is a major stimulator of $VEGF_A$ expression (Shweiki *et al.*, 1992). This process involves the binding of hypoxia-inducible-factor-1 (HIF-1) to a HIF-1 binding site located in the $VEGF_A$ promoter (Levy *et al.*, 1995, Liu *et al.*, 1995). HIF-1 increases the level of transcription and stabilises VEGF mRNA by increasing its half life via a mechanism involving protein binding to sequences located in the 3' untranslated region (UTR) of the $VEGF_A$ mRNA (Stein *et al*, 1995, Damert *et al*, 1997, Claffey *et al*, 1998). One of the stabilising proteins identified is HuR mRNA binding protein (Levy *et al*, 1998).

Alterations in tumour suppressors leads to an elevation of $VEGF_A$ in tumour cells and loss of wild type p53 is associated with an increase in angiogenesis in developing tumours (Vanmeir *et al*, 1994). Wild type p53 was identified as an inhibitor of $VEGF_A$ production (Mukhopadhyay *et al*, 1995) and mutated p53 can potentiate $VEGF_A$ expression (Kieser *et al*, 1994, Takahashi *et al*, 1998). Therefore in tumours with mutated p53 there is an increased number of blood vessels and an increased potential for growth and spread.

1.3.5 $VEGF_A$, cancer and CRC

Different blood vessels in the human body are thought to have varying degrees of permeability. In mice, guinea pigs and hamsters, blood vessels lining the peritoneal cavity have a greater permeability due to increase in vascular permeability factor VPF; the original name for $VEGF_A$ (Senger *et al.*, 1983).

Tumours demonstrate increased vascularity and angiogenesis (Ide *et al.*, 1939, Algire, 1945) thought to be due to angiogenic factor and when investigated using bovine pituitary follicular cells, known to have increased mitogenic activity. Increased mitogenic activity was thought to be due to a secreted protein that was freely diffusable (Ferrara *et al.*, 1987, Ferrara & Henzel, 1989). The protein responsible was later identified as $VEGF_A$ (Plouet *et al.,* 1989, Connolly *et al.*, 1989, Leung *et al.*, 1989, Keck *et al.*, 1989, Conn *et al.*, 1990).

The demonstration of the crucial role of $VEGF_A$ in tumour angiogenesis came from the use of anti-$VEGF_A$ antibodies which when injected into tumour-bearing nude mice resulted in decreased tumour growth (Kim *et al.*, 1993). In addition mutant receptor

KDR/Flk-1 or the presence of anti-Flk-1 antibodies also decreased tumour growth (Millauer *et el.*, 1994, Asano *et al.*, 1995, Millauer *et al.*, 1996, Borstrom *et al.*, 1998). An alternative method to investigate the effect of blockade of $VEGF_A$ on tumour angiogenesis came with fluorescent microscopy enabling the visualisation of the tumour vasculature. Anti-$VEGF_A$ treated animals had almost complete suppression of tumour angiogenesis compared to controls (Borgstrom *et al.*, 1996). Cancers in which $VEGF_A$ is thought to be involved in the pathogenesis is shown in Table 1.1.

Tumours	**References**
Glioblasoma	Millauer *et al.*, 1994
Head & Neck	Teknos *et al.*, 2002 Smith *et al.*, 2000
Insulinoma	Inoue *et al.*, 2002
Haematological malignancies	Gerber *et al.*, 2003
Cervix	Guidi *et al.*, 1995
Endometrial	Giatromanolaki *et al.*, 2001
Breast	Toi *et al.*, 1995a, 1996 Linderholm *et al.*,1998
Melanoma	Erhard *et al.*, 1997
Gastric	Maeda *et al.*, 1996 Saito *et al.*, 1999
Renal Cell	Iliopoulos *et al.*, 1996
Lung	Imoto *et al.*, 1998
Liver	Li *et al.*, 1998 Ng *et al.*, 2001

Table 1.1
A summary of some of the cancers in which $VEGF_A$ is thought to be involved in tumour pathogenesis.

As higher levels of $VEGF_A$ protein expression is observed in adenomas and CRC compared to normal bowel mucosa, $VEGF_A$ may have some role to play in the development and progression of CRC (Wong *et al.*, 1999, Andre *et al.*, 2000). Interestingly as the difference in levels of $VEGF_A$ between adenomas and CRC was small upregulation of $VEGF_A$ might only occur early in the development of CRC (Hanrahan *et al.*, 2003).

Cyclo oxygenase 2 (COX_2) is known to be involved in the angiogenesis of CRC (Sheng *et al.*, 1997, Tsujii *et al.*, 1998). Studies on cell lines have shown that alteration in COX_2 had corresponding alterations in $VEGF_A$ (Tsujii *et al.*, 1998). $VEGF_A$ expression also correlated with the mean vessel density in CRC supporting the hypothesis that $VEGF_A$ plays some role in CRC angiogenesis (Nakasaki *et al.*, 2002, Tsuji *et al.*, 2002).

In CRC $VEGF_A$ concentration is found in the cytoplasm of cells and to be highest at the invasive edge of the tumour (Ono *et al.*, 2000). Levels of $VEGF_A$ correlated with tumour size, blood vessel involvement, histological type and probably with Duke's stage (Fukisaki *et al.*, 1998, Baker *et al.*, 2000, Lee *et al.*, 2000, Nakasaki *et al.*, 2002, Hanrahan *et al.*, 2003).

The soluble isoforms, $VEGF_{121}$ and $VEGF_{165}$, both gene transcription (mRNA) and protein expression have been identified in CRC and are believed to be the more important isoforms involved in CRC pathogenesis (Uthoff, 2002). Anti Flk-1 antibodies inhibit tumour angiogenesis and endothelial cell survival in colon cancer carcinomatosis (Shaheen *et al.*, 2001). Flt-1 gene transcription correlate with tumour

grade, Duke's stage and lymph node involvement whilst Flk-1/KDR correlate with lymph node involvement (Hanrahan *et al.*, 2003).

CRC have reduced $VEGF_A$ expression (Wynter *et al*, 1999) and COX_2 protein expression (Karnes *et al.*, 1998) was found in MSI-H tumours compared to MSS. These observations suggest that MSI-H tumours have decreased levels of angiogenesis which may help to explain why these tumours demonstrate decreased distant spread and a better prognosis.

In addition to angiogenesis, tumour growth and spread is also dependant on lymphangiogenesis, a process controlled by another of the VEGF family of cytokines, $VEGF_C$ which is discussed in the next section.

1.4 Vascular Endothelial Growth Factor C

1.4.1 Lymphangiogenesis

Until recently little was known about the lymphatic system and its mechanism of development and control but new animal models have helped to appreciate the functions of the lymphatic system in maintaining plasma volume and tissue pressure, and its important role in the immune system (Boardman and Swartz, 2003).

The lymphatic system is made up of vessels starting as an open ended transport system in interstitial spaces. Protein rich fluid extravasates from blood into the interstial tissue and is collected by the open capillaries which drain into progressively larger lymphatic vessels. The collecting lymphatics then drain into the thoracic duct and finally into the left subclavian vein. On the right side the lymphatics drain into the right lymphatic duct and then into the right subclavian vein. During its' pathway to the central circulation lymph drains through at least one lymph node where presentation of antigens and filtering can occur (Baldwin *et al.*, 2002). A schematic of the lymphatic system is shown in Figure 1.9.

In the embryo lymphatics develop from blood vessels and endothelial cells originate from the cardinal vein. Additional sources of lymphatic endothelium are from lymphangioblasts and lymphatic endothelial precursor cells (Alitalo *et al.*, 2002, Wilting *et al.*, 2003, Salven *et al.*, 2003).

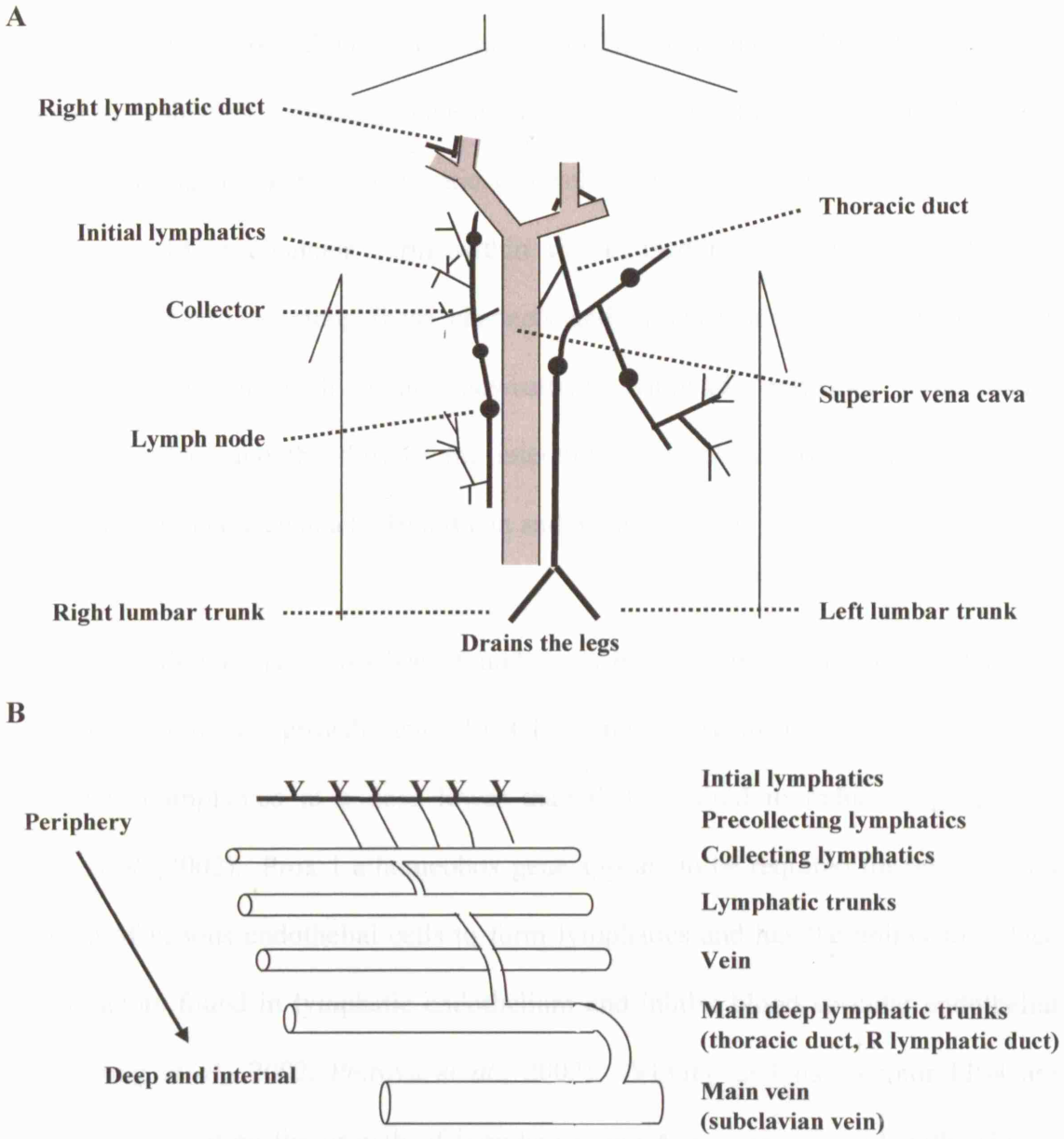


Figure 1.9
Schematic of lymphatic vascular tree modified Baldwin *et al.* (2002). **A:** Passage of lymph through the lymphatics and drainage into venous circulation. **B:** Schematic representation of the vessels of the lymphatic system.

A new animal model of lymphatic development has revealed new mechanisms of lymphangiogenesis (Boardman and Swartz, 2003). This model looked at different markers in evaluating lymphangiogenesis: fluid channel markers, cellular markers and soluble molecular markers. Lymphatic channel formation is related to the routes of fluid flow. Fluid channels form within the extracellular matrix and lymphatic endothelial cells move along these channels and organise into functional networks which are continuous with up and downstream lymphatic networks. The matrix metalloproteinases and the flow of the interstitial fluid are factors which affect the direction of lymphatic channels (Boardman and Swartz, 2003).

Molecular mediators have also been found to be involved in the development of the lymphatics. Fibroblast growth factor-2 (FGF-2) induced lymphangiogenesis in mouse cornea when implanted at a dose lower than that required to induce angiogenesis (Chang *et al.*, 2002). Prox 1 a homeobox gene appears to be required for budding and sprouting of venous endothelial cells to form lymphatics and has the ability to induce growth factors found in lymphatic endothelium and inhibit blood vascular endothelial genes (Hong *et al.*, 2002, Petrova *et al.*, 2002). $VEGF_C$ and its receptor Flt-4 are involved in regulating the growth of lymphatics and over expression of both $VEGF_C$ and Flt-4 independently results in hyperplasia of the lymphatic vessels (Jeltsch *et al.*, 1997, Veikkola *et al.*, 2001). Currently there is much debate as to whether mechanical signals such as flow stimulate expression of lymphatic growth factors or growth factors that instigate lymphatic flow.

Lymphangiogenesis and Cancer

It has been suggested that lymphangiogenesis plays a role in the spread of a tumour but it is uncertain whether it has a role in tumour growth. Lymphatics are thought not to develop in tumours due to the intra-tumour pressure causing the lymphatic vessels to collapse (Stacker *et al.*, 2002), although there is evidence however, that the level of lymphatic factors correlate with spread of the primary tumour (Stacker *et al.*, 2002) (Figure 1.10).

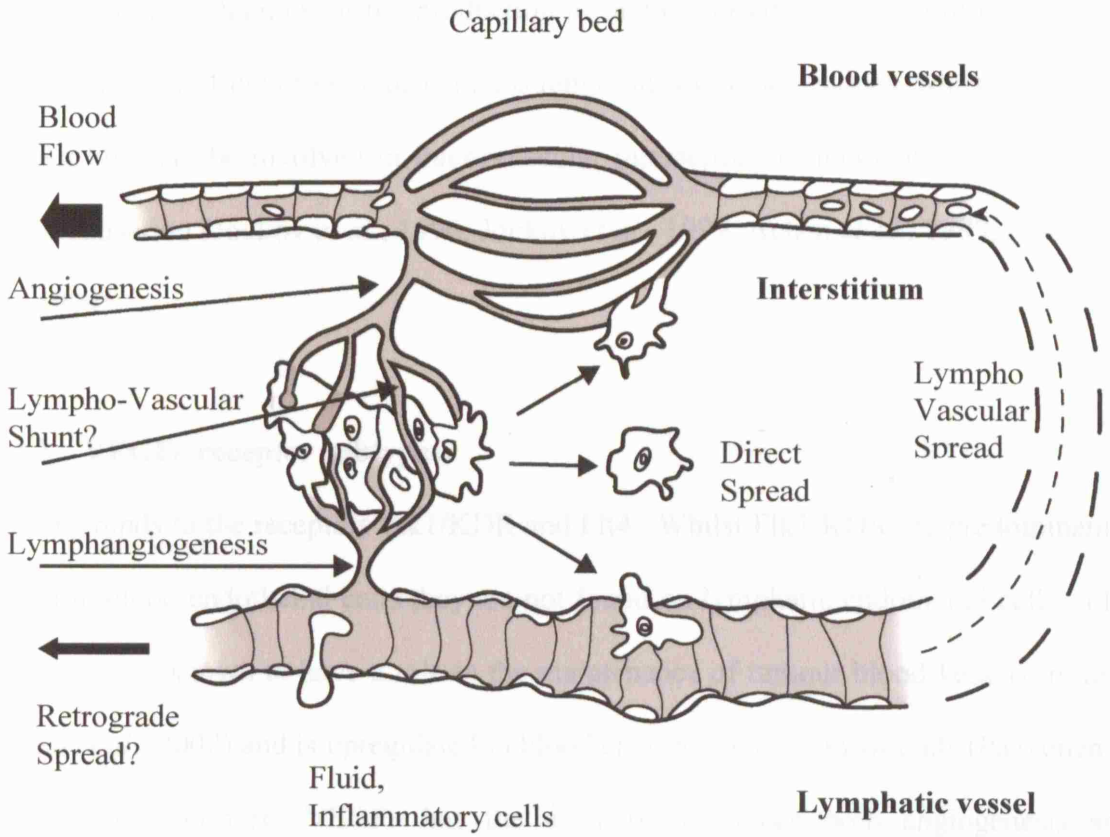


Figure 1.10
Schematic of lymphatic system and spread of tumour cells, modified from Stacker *et al.* (2002).

1.4.2 Molecular Structure of $VEGF_C$

$VEGF_C$'s chromosomal location is 4q34.1-q34.4 and the protein is a disulphide linked prepropeptide of M_r 59-61kD, with many similarities to $VEGF_D$. $VEGF_C$ is conserved at the amino acid level but it has the propepetides at the N- and C- termini of the conserved VEGF homology domain (VHD). VHD is the region on the gene that contains receptor binding sites. $VEGF_C$ is secreted as a propeptide and in order to be functionally active it is proteolytically cleaved to the mature peptide. The stepwise cleavage increases the affinity of $VEGF_C$ for the receptors Flk1/KDR and Flt4 (Baldwin *et al.*, 2002). A diagram of the production of mature $VEGF_C$ is shown in Figure 1.11. The C terminal of the propeptide contains numerous cysteine residues, whose role is not known but may be involved in intermolecular interactions to modulate bioavailability and localisation (Joukov *et al.*, 1996, Joukov *et al.*, 1997, Achen *et al.*, 1998, Stacker *et al.*, 1999).

1.4.3 $VEGF_C$ receptor subtypes

$VEGF_C$ binds to the receptors Flk1/KDR and Flt4. Whilst Flk1/KDR are predominantly found in blood endothelial cells they are not found on lymphatic endothelial cells. Flt4 has also been shown to have a role in the maintenance of tumour blood vessels in mice (Kubo *et al.*, 2000) and is upregulated in blood endothelial cells in wounds (Paavonen *et al.*, 2000). Therefore, $VEGF_C$ has the capacity to induce both angiogenesis and lymphangiogenesis.

Flt4 is thought to be the major regulator of lymphangiogenesis. In embryos Flt4 is expressed in all vessels but during development the expression of Flt4 in blood vessels

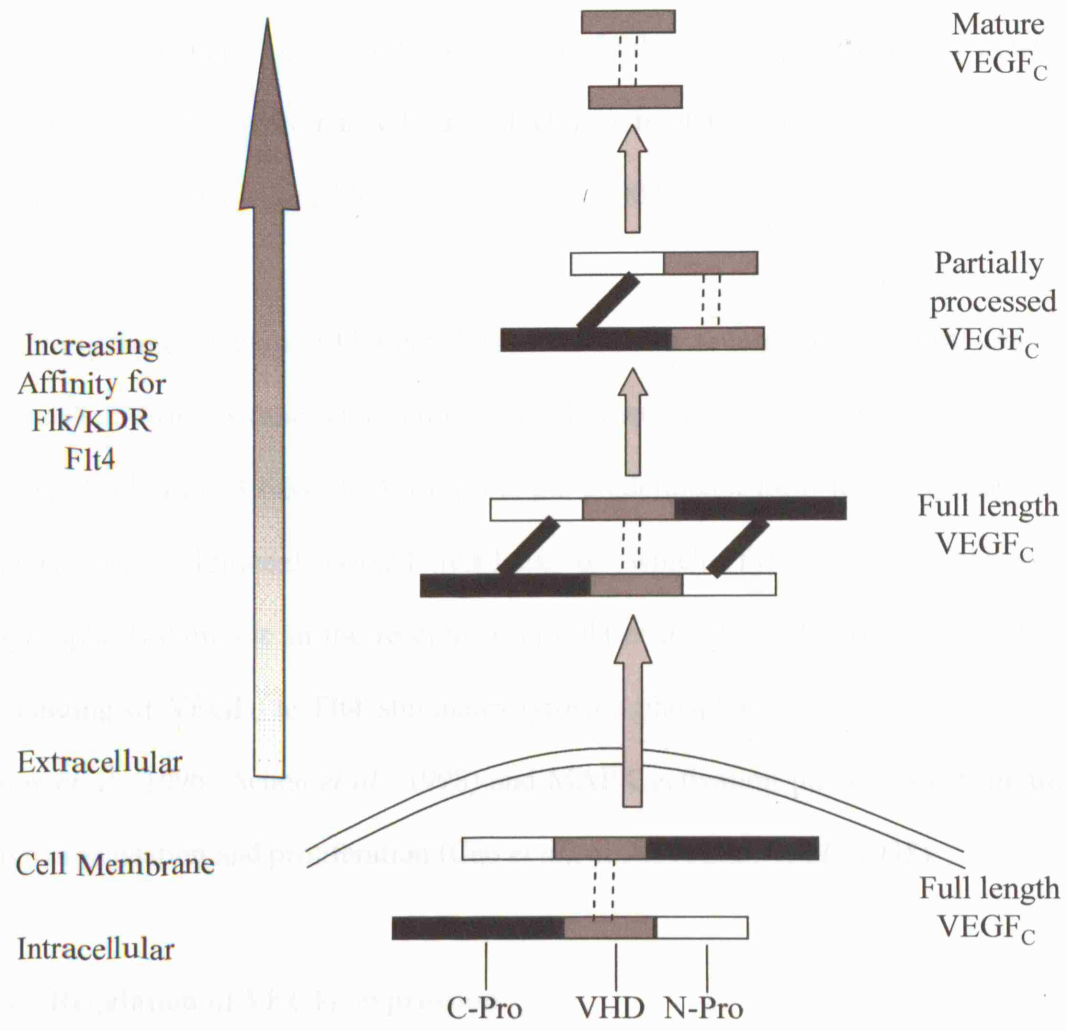


Figure 1.11
Schematic of the production of mature VEGF$_C$. VEGF$_C$ is secreted inan inactive form, it is then proteolytically cleaved to become an active mature protein; modified from Baldwin *et al*. (2002).

decreases while its expression on lymphatic endothelium is maintained (Kaipainen *et al.*, 1995). Flt4 has also been found to be expressed on non-endothelial cells such as monocytes, tumour associated macrophages and immature dendritic cells (Fernandez *et al.*, 2001, Schoppman *et al.*, 2002). A recent study demonstrated that corneal dendritic cells express $VEGF_C$ in normal cells and which is upregulated in inflammation (Mimura *et al.*, 2001, Cursiefen *et al.*, 2002, Hamrah *et al.,* 2003).

The chromosome location of Flt4 is 5q35 and it has two isoforms Flt4s (short) and Flt4l (long) which differ in caboxyl termini due to alternate mRNA splicing (Pajusola *et al.*, 1993, Galland *et al.*, 1993). Flt4l (long) is the predominant form found in tissues and contains three additional tyrosyl residues, of which Tyr1337 is an important autophosphorlyation site in the receptor (Pajusola *et al.*, 1993, Fournier *et al.*, 1996). The binding of $VEGF_C$ to Flt4 stimulates tyrosine phosphorylation (Lee *et al.*, 1996, Joukov *et al.*, 1996, Achen *et al.*, 1998) and MAPK activation, increases cell motility, actin reorganisation and proliferation (Cao *et al.*, 1998, Joukov *et al.*, 1998).

1.4.4 Regulation of $VEGF_C$ expression

As the main regulator of lymphangiogenesis $VEGF_C$, unlike $VEGF_A$, is not induced by hypoxia, Ras or mutant p53. There are however, several inducers of $VEGF_C$ which include IL-1alpha, IL-1ß, TNF-alpha, TGF-ß, IGF-IR, EGF, PDGF and PI3K. Induction of $VEGF_C$ mRNA in the serum seems to be independent of protein synthesis (Enholm *et al.*, 1997, Ristimaki *et al.*, 1998, Tang *et al.*, 2003).

1.4.5 $VEGF_C$, cancer and CRC

The over expression of $VEGF_C$ in experimental models using tumour cell lines increases lymphangiogenesis and angiogenesis resulting in increasd tumour growth, intra-tumoural lymphatics, peritumoural lymphatics, the recruitment of macrophages and metastatic spread of the primary tumour to regional lymph nodes (Karpanen *et al.*, 2001, Mandriota *et al.*, 2001, Skobe *et al.*, 2001, Yanai *et al.*,2001, Mattila *et al.*, 2002).

Similar results have been found in clinical trials. For example, in head and neck squamous cell carcinomas which predominantly metastasise to regional lymph nodes, there was a correlation between tumour $VEGF_C$ expression and recurrences, and larger tumours demonstrated more $VEGF_C$-positive vessels (Neuchrist *et al.*, 2003). A positive correlation between $VEGF_C$ and lymph node metastasis was also found in breast cancer and furthermore, patients whose tumours were negative for $VEGF_C$ protein expression had a significant increase in disease-free survival (Nakamura *et al.*, 2003). Gastric tumours expressing alpha fetoprotein are known to have a poorer prognosis and a higher incidence of liver metastasis. These tumours were also shown to have an increased expression of $VEGF_C$ so the potential for increased lymphangiogenesis (Chang *et al.*, 1992, Kamei *et al.*, 2003). In pancreatic endocrine neoplasm's, $VEGF_C$ had low to moderate expression in the primary tumour, but liver metastases demonstrated significantly increased $VEGF_C$. There was increased expression also of the receptors Flk1/KDR and Flt4 in the majority of neoplastic cells, perhaps indicating that $VEGF_C$ up-regulation was important in the tumour progression and metastases (Hansel *et al.*, 2003). Similar results have been found in non-small-cell lung cancer (Kajita *et al.*, 2001). In contrast, $VEGF_C$ expression was shown to correlate only with histological grade of squamous cell carcinoma of the oesophagus

(Noguchi *et al.*, 2002). A summary of the relationship between $VEGF_C$ and cancers reported to date is shown in Table 1.2.

Tumour	**Role of $VEGF_C$ in tumour spread**	**Reference**
Thyroid	Lymph node invasive tumours	Bunone *et al.*, (1999)
Gastric	Lymph node invasive tumours	Yonemura *et al.*, (1999)
	Lymphatic and venous invasion	Ichikura *et al.*, (2001)
Prostate	Lymph node positive tumours	Tsurusaki *et al.*, (1999)
Lung	Lymph node metastasis	Niki *et al.*, (2000)
CRC	Lymph node metastasis, lymphatic Involvement and depth of invasion	Akagi *et al.*, (2000)
Cervical	Pelvic lymph node metastasis	Hashimoto *et al.*, (2001)
Oesophageal	Tumour invasion, tumour stage, venous invasion, lymphatic and lymph node invasion	Kitadai *et al.*, (2001)
Head & Neck	Cervical lymph node	O-charoenrat, *et al.*, (2001)
Breast	Lymphatic vessel invasion	Kinoshita *et al.*, (2001)

Table 1.2
Correlation of $VEGF_C$ expression with different tumours.

$VEGF_C$ and colorectal cancer

$VEGF_C$ expression has been found to be increased in CRC compared to normal tissue, and was lower in adenomas compared to carcinomas. This may suggest a role of $VEGF_C$ in advanced disease when it is more likely to metastasise, evidenced by a recent observation that $VEGF_C$ gene expression correlated significantly with tumour grade and size (Hanrahan *et al.*, 2003). Furudoi *et al.* (2002) found that at the deepest invasive site of a CRC, $VEGF_C$ protein expression correlated with Duke's stage and these cancers had a significantly poorer prognosis (Akagi *et al.*, 2000) and both $VEGF_C$ mRNA and protein expression correlated with lymph node metastasis and lymphatic invasion (Kawakami *et al.*, 2003).

The above observations suggest a potential role for $VEGF_C$ in the development and progression of CRC. However, there has been little if any investigation of the relationship between MSI and $VEGF_C$. As MSI-H tumours have decreased tumour spread and decreased lymphatic involvement and an improved prognosis the role of $VEGF_C$ in MSI would be of interest.

CHAPTER 2

AIMS AND EXPERIMENTAL DESIGN

CHAPTER 2

2.1 Overview of scientific question

For a CRC to develop several genetic alterations are required and these may occur via essentially two main pathways. The first, referred to as the 'classical pathway' and thought to be the process by which the majority of tumours develop, involves the LOH of 5q, 17p and 18q (Fearon & Vogelstein *et al.*, 1990, Cho & Vogelstein, 1992). These alterations result in mutation of genes that affect maintenance of normal cellular function such as the APC (Boomer *et al.*, 1986), p53 (King *et al.*, 2000) and SMAD (Fodde *et al.*, 2001) genes as well as Kras (Chung, 2000).

The second, or 'alternative', pathway is thought to be involved in the development of approximately 15% of CRC and is characterised by MSI. In MSI, there is an increased susceptibility to acquiring mutations in genes as a result of lost or reduced function of the MMR genes that are responsible for correcting errors that occur in DNA during replication. Genes most susceptible to such MMR errors are those containing regions of repeated nucleotide bases and include for example, TGFβ, and Bax (Goel *et al.*, 2001, Jass *et al.*, 2002). The identification of MSI cancers is made by analysis of the number of mutations found within a series of specific mononucleotide and dinucleotide(regions of DNA), and tumours are classified as MSI-H when demonstrating ≥30-40% of markers mutated, MSI-L <30-40%, or MSS when no markers are mutated (National Cancer Institute, 1997, Boland *et al.*, 1998).

MSI-H tumours have a low incidence of LOH but show mutations of the MMR genes MLH1 and MSH2 (Markowitz *et al.*,1995, Fujiwara *et al.*,1998). These tumours are

poorly differentiated have an increased number of intra- and peri-tumour lymphocyte infiltration and typically more are mucinous cancers (Jass *et al.*, 1998, Young *et al.*, 2001). In contrast, MSI-L tumours have LOH of chromosome 1p and increased Kras mutations, and show equivocal incidence of p53 and APC mutations as MSS tumours (Konishi *et al.*, 1996). In addition to differing underlying genetic alterations, MSI-H tumours have a different phenotype to MSI-L and MSS tumours; with decreased lymphatic involvement and distant spread (Tomlinson *et al.*, 2002). More significantly however, patients with MSI-H have an improved prognosis and survival compared to MSI-L and MSS tumours (Gryfe *et al.*, 2000, Wright *et al.*, 2000). MSI-L tumours have no definite clinicopathological phenotype but tend to be commoner in the left colon or rectum (Jass *et al.*, 1999) and have a more aggressive phenotype with increased risk of metastasis (Jass *et al.*, 1999), and early tumours have a high risk of recurrence (Kambara *et al.*, 2001).

Overall, MSI-H tumours have a favourable phenotype compared to other CRC and as such, are of particular clinical interest. Furthermore, these observations highlight the need to be able to clarify the differences between the MSI sub-groups, and to identify factors of potential use for diagnostic and prognostic purposes. However, the precise mechanisms that underlie the differences in MSI remain unclear.

Central to tumour development, growth, and spread are the processes of angiogenesis (Carmelieit & Jain, 2000) and lymphangiogenesis (Stacker *et al.*, 2000). To consider each individually; a cluster of cancer cells can only grow a few millimetres unless angiogenesis is activated to allow further development and growth. One of the key proteins involved in this process is VEGF which becomes stimulated in response to

developing hypoxia (Schweiki *et al.*, 1992, Adamis *et al.*, 1994, Aiello *et al.*, 1994) as the tumour grows, the resultant angiogenesis allowing more oxygen to be available and so permitting further tumour development and growth (Hanahan & Folkman, 1996). However, and unlike in normal tissue, the resulting blood vessels that develop within tumours are irregularly shaped, tortuous, dilated and maintain an ability to constantly grow (Bergers & Benjamin, 2003). Adenomas and CRC tumours have been found to have increased levels of VEGF protein compared to normal colonic mucosa (Wong *et al.*, 1999, Andre *et al.*, 2000), and VEGF protein expression would seem to correlate with tumour size, blood vessel involvement, histology and Duke's stage (Hanrahan *et al.*, 2003). In addition, protein COX_2 which is also involved in angiogenesis, has been found to have increased levels in CRC (Sheng *et al.*, 1997, Tsujii *et al.*, 1997). Furthermore, that angiogenic capacity is relevant in MSI tumours is clear from the reported observations that in contrast to MSS tumours, MSI-H tumours have decreased levels of VEGF (Wynter *et al.*, 1999) and COX_2 proteins (Karnes *et al.*, 1998).

In addition to angiogenesis, lymphangiogenesis enables the spread of tumour cells (Stacker *et al.*, 2002). The protein $VEGF_C$ and its receptor Flt4 have been demonstrated to induce both angiogenesis and lymphangiogenesis, and in cancer both have been shown to increase the intra- and peri- tumoural lymphatics, and recruitment of macrophages (Karpanen *et al.*, 2001, Mandriota *et al.*, 2001, Skobe *et al.*, 2001, Yanai *et al.*, 2001, Mattila *et al.*, 2002). Furthermore, both are increased in lymph nodes and metastasis (Neuchrist *et al.*, 2002). A higher level of $VEGF_C$ has been found in CRC compared to adenomas and to advanced cancers (Hanrahan *et al.*, 2003). Furthermore, both $VEGF_C$ protein and mRNA have been reported to correlate with lymph node involvement, lymphatic invasion and metastasis (Akagi *et al.*, 2000).

The processes of angiogenesis and lymphangiogenesis have thus, been shown to be directly related to tumour growth, development and spread. Some of the fundamental molecules involved in the mediation of these processes are the VEGF isoforms VEGF and $VEGF_C$. To date however, little is known of the relationship between VEGF, $VEGF_C$ and CRC with MSI.

2.2 Hypothesis

The central hypothesis of this thesis therefore, is that the difference in clinical presentation and prognosis of CRC with MSI may be explained by a differing underlying angiogenic and lymphangiogenic potential, which in turn, is mediated by differences in the production and activity of the VEGF isoforms VEGF and $VEGF_C$. Furthermore, should VEGF and $VEGF_C$ differ significantly between MSI groups, these may serve to help diagnostic and prognostic evaluation of CRC.

2.3 Aims and Experimental Design

The aim of this study was to investigate the two VEGF genes, VEGF and $VEGF_C$, in CRC of MSI-H, MSI-L and MSS phenotypes, and as a potential mechanism underlying the differences in phenotypic and prognostic presentation.

To address these aims, the following had to be undertaken to;

1. Identify and collect a series of CRC samples and determine MSI status
2. Confirm that these samples were a true representation of MSI in CRC
3. Determine the activity of the genes for VEGF and $VEGF_C$ in relation to MSI by measurement of the respective levels of gene transcription (mRNA), and

4. Determine the levels of VEGF and $VEGF_C$ protein products in relation to MSI by measurement of a). blood vessel density as a measure of angiogenesis and indirect measure of VEGF protein production, and b). the levels of $VEGF_C$ protein production as an indication of lymphangiogenic potential.

Experimental design

Each aim above was addressed as follows;

1. Collection of samples and determining MSI

Although samples may be obtained prospectively, to obtain a suitable number for analysis can be a prohibitively time-consuming process that limits the extent of analysis possible within a limited time-frame. However, for the studies included within this thesis a series of CRC archival samples previously embedded in paraffin wax and whose MSI status had been determined in an earlier study, were readily available, and were selected for analysis. The advantage of use of this archival of wax samples was that it allowed a large sample size to be available for analysis. Furthermore, accurate data collection and follow up of patients from whom the archival samples were derived would enable additional data about demographics and outcome to be obtained. However, although demographic information was available for these samples, the outcome of patients from which the samples were obtained in this thesis was unknown.

The usage of paraffin wax-embedded blocks as opposed to fresh samples required an alteration in experimental design. Gene transcription was analysed by examining the levels of mRNA of each specific gene, and was undertaken by RT-PCR. Under normal circumstances (for example, using fresh, or frozen tissue), gene mRNA products are

examined by amplifying regions of the gene in question that are typically 200-300 bp in length. However, extraction of RNA from wax embedded samples yields much shorter lengths of total cellular RNA. As such, the RT-PCR protocol required modification, and specific primers (used to amplify the required region of mRNA) needed to be developed and tested that amplified much shorter regions of mRNA than normal. In order that the genes under investigation are successfully amplified, mRNA sequences of a length around 100bp were chosen. These used in conjunction with a 'step up' PCR cycling protocol permitted an optimum amplification of the genes under investigation; presented in Materials and Methods, Chapter 3.3.

2. Samples as a true representation of MSI in CRC

Prior to commencing experimental analysis, the sample group was first analysed to verify that it was representative of the overall pattern of CRC in the population, and of MSI in CRC. This involved the MSI phenotype being analysed for gender and age of the patient, and site and Duke's stage of the tumour. The Duke's stage was analysed according to a routine protocol by an NHS pathology laboratory. The analysis of demographics is presented in Chapter 4.

In addition to analysing the demographics, each tumour sample required verification for its location within the wax-embedded blocks. RT-PCR is a very sensitive technique and it was important to ensure that the tumour was being analysed and not 'contaminated' by any normal colonic tissue which may present differing levels of gene transcription and protein expression. Initial staining with Haematoxylin and Eosin was undertaken to identify the tumour, and then where necessary, samples were re-aligned in new blocks so that cut, serial sections were of tumour tissue free of any associated

normal tissue and were used to provide material for analysis of gene transcription and protein expression, the results of this are presented in Chapters 6 and 7.

3. Analysis of gene transcription

Following determining that the samples were representative of CRC and MSI, and the location of the tumour was identified, an analysis of gene transcription of the samples was undertaken. Although MSI has been demonstrated to cause an increase in the mutational rate of genes, the investigation of gene transcription would help identify any role that alterations in gene activity may play in MSI and the development of CRC. VEGF is a key mediator of angiogenesis and required for the development of CRC. VEGF is produced in various isoforms, the storage form $VEGF_{189}$, $VEGF_{206}$, and the soluble isoforms that are thought to be the principal functional mediators that stimulate angiogenesis $VEGF_{121}$ and $VEGF_{165}$. It was important therefore, to analyse the transcription specifically of the two soluble isoforms, $VEGF_{121}$ and $VEGF_{165}$. The first section of Chapter 5 investigated whether MSI has an effect on the levels of $VEGF_{121}$ and $VEGF_{165}$ gene transcription, and which therefore, may help account for the different phenotype of MSI tumours.

Lymphangiogenesis is also important for the development of CRC, of which the VEGF isoform $VEGF_C$ is believed to be a crucial mediator. The second section of Chapter 5 evaluated the relationship between MSI and transcription of $VEGF_C$ in CRC. Currently, $VEGF_C$ is believed to consist of a single isoform, and so to obtain a representation of the levels of transcription of the whole gene, separate analyses were made of transcription from the 5-primer (5'; front) and 3-prime (3'; back) regions of the $VEGF_C$ gene.

The third section of Chapter 5 determined whether there was a difference in gene transcription in relation to patient demographics and that of CRC.

Throughout, the levels of gene transcription were analysed by semi-quantitative RT-PCR. Primers were designed to detect small fragments of mRNA and to cross at least one exon-exon boundary to ensure that only mRNA was amplified and not any potentially contaminating genomic DNA sequences. Following optimisation of the PCR reaction, a 'step-up' PCR cycling protocol was found to give optimal results. PCR products were analysed by agarose gel electrophoresis, and the band intensity of specific gene products measured by scanning densitometry, and the relative levels of gene transcription determined as a ratio of the integrated optical density (IOD) of the specific gene product and that of the internal house keeping gene, glyceraldehyde 3 phosphate (GAPDH 3). The internal housekeeping gene was used, as theoretically it should be expressed at a constant rate to allow normalisation of results and correct for a multitude of factors such as altered basal transcription rates, metabolisation, proliferation and concentration of starting template.

4. Analysis of protein expression

In order to investigate the MSI phenotype further, it was necessary to examine the end products of gene transcription, i.e. production of the functional protein product. For example, whilst levels of gene transcription may be altered, if there is no associated change in protein product, it is unlikely that these alterations in gene transcription have a role to play in the disease process. Furthermore, gene transcription may be unaltered but for example protein production or half-life may become changed, and which has some effect on the disease process. Chapters 6 and 7 present the investigation of

protein expression and evaluate any correlation with gene transcription, tumour site, or Duke's stage.

Protein expression was analysed by immunohistochemistry. The VEGF isoforms $VEGF_{121}$ and $VEGF_{165}$ are soluble isoforms and so any measurement of their levels in fixed tissue would not give a true indication of their levels of production. As such, using immuno-histochemistry to detect VEGF protein levels in the fixed samples used would not provide an appropriate analysis, or a correct comparison with the levels of gene transcription. However, analysing the tissue blood vessel density serves as a good indicator of the level of angiogenesis. Therefore, as an indirect measurement of the production of the soluble VEGF isoforms $VEGF_{121}$ and $VEGF_{165}$ that mediate angiogenesis, immuno-histochemistry was used with an antibody against to CD34 to detect blood vessels; this was as recommended by the NHS pathology laboratory, as provides a more specific marker for blood vessels than lymphatic vessels. Blood vessel density was then recorded by examination under light microscope.

Lymphangiogenesis was evaluated indirectly by the measurement of levels of $VEGF_C$ protein as detected by immuno-histochemistry and an antibody to the $VEGF_C$ protein. The slides when examined under a light microscope to determine whether they were positively or negatively stained for $VEGF_C$.

The materials and methods used for this thesis are described in detail in Chapter 3.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

3.1 Patient Selection

Ethics approval was gained from the joint UCL/UCLH Ethics Committee on Human Research.

Archival wax-embedded samples of colorectal cancer were used. All patients whose samples were used for analysis were known not to have developed another large bowel cancer in seven years after the primary cancer and so are regarded as sporadic CRC. All samples were prepared by a NHS histology laboratory according to standard protocols.

Eight samples of normal colon were obtained from patients having resections for colon cancer. These samples were fixed in formalin for 24 hours and then stored in 70% alcohol, before being embedded in paraffin wax blocks, according to a standard protocol.

3.2 Overview of analysis

3.2.1 Duke's Classification

Tumours were classified according to Duke's Classification by a Consultant Pathologist.

3.2.2 Microsatellite status

The MSI status of tumours had been determined and classified by another researcher in the Colorectal Department and as reported (SenGupta *et al.*, 1997). Single stranded

conformational polymorphism (SSCP) was used to identify unstable loci found in MSI, using mononucleotide markers BAT25, BAT26 and BAT40 and the dinucleotide markers were D5S346, D2S123, and D17S250. Tumours were classified as MSS if none of the 6 loci were unstable, MSI-L if one loci was unstable, or MSI-H if two or more loci were unstable (SenGupta *et al.*, 1997).

3.2.3 Data Analysis

The levels of gene transcription and protein expression were compared according to gender, age of patient, site of tumour and Duke's stage. Analysis of age was analysed as below and above 70 years due to this being the median age at diagnosis for CRC from the Thames cancer registry from which these samples were obtained. Tumours were then analysed according to MSI status, and whether mutations were present in mononucleotide or dinucleotide markers. The results were analysed according to which type of marker was mutated as Jass *et al.* (2002) has provided evidence that true MSI-L tumours are those with only mutated dinucleotide markers.

3.3 Analysis of gene expression

3.3.1 RNA Isolation

Total cellular RNA was extracted from two 20μm sections of each wax-embedded CRC and by an adaptation of the protocol for the paraffin block RNA isolation kit (Ambion Inc., Texas, USA). The sections were dewaxed by adding 1ml of xylene (BDH Lab Supplies) and then agitated for 20 minutes on a rotating platform (Titertek, Flow labs). The samples were centrifuged at 12,000 x g for 5 minutes and the xylene was discarded. The samples were dehydrated by washing 3 times with 1ml of 100% ethanol (BDH Lab

Supplies) agitating for 3 minutes at room temperature with each wash. The final ethanol wash was removed and the samples were allowed to air dry for 5 minutes.

To digest the tissue, 5µl proteinase K and 100µl proteinase K digestion buffer (Ambion Inc, USA) were added to each sample which were then incubated in a water bath (Grant, Prior Labs Ltd, UK) for 2 hours at 45°C. During the 2 hours of incubation samples were occasionally agitated on the rotating platform. Next 600µl RNA extractaze buffer was added before vortexing (Vortex Genie 2, Scientific Industries) for five, 5 sec bursts. Samples were then incubated at room temperature for 5 minutes before guanidinium based extractaze buffer was added to solubilise the tissue, extricate the RNA and inactivate any RNAses.

For isolating RNA 700µl of acid phenol chloroform was added followed by vortexing for five, 5 sec intervals. The samples were incubated at room temperature for 5 minutes and then centrifuged at 12,000 x g for 7 minutes. This process differentiates the RNA from the DNA and protein and three layers develop as a result due to the different relative densities of the components of the sample. The bottom layer is the organic phase containing proteins dissolved in acid phenol chloroform. The interphase contains DNA and the upper aqueous phase contains soluble RNA which was transferred to a fresh 1.5ml Eppendorf tube (Starsted, UK).

The samples were mixed briefly after adding 1µl of linear acrylamide which acts as a carrier. An equal volume of isopropanol (Sigma,UK) was added and mixed by inversion before incubation at -20°C overnight to precipitate out the RNA. Following centrifugation at 12,000 x g for 15 minutes the isopropanol was removed and the samples washed in 500µl of cold 75% ethanol by vortexing before spinning at 12,000 x

g for 15 minutes. Finally the ethanol was removed and the RNA pellet allowed to dry for 5 minutes at room temperature before it was resuspended in RNA storage solution (Paraffin Block RNA Isolation Kit, Ambion Inc., Texas, USA).

Typically the RNA extracted from paraffin wax blocks was no greater than a few 100bp in length. Therefore the primers for the RT-PCR were designed to amplify a product of this specification.

Spectrometry

The concentration of extracted RNA was determined by measuring the light absorbance of nucleotides in solution at the ultraviolet region of the spectrum i.e. from a 260nm and 280nm wavelength. A spectrophotometer (UV 1101 Biotech Photometer, WPA, UK) was used and the samples were quantified for RNA using the Beer-Lambert Law:

$$A_\lambda = \varepsilon_\lambda bC$$

A_λ - the samples's absorbance value at 260nm

ε_λ - absoptivity coefficient of the material at specific wavelength

b - the path-length through the sample

C - concentration of the sample

3.3.2 Primer Design

Primers for PCR are oligonucleotides that determine the size, the sequence location of the PCR product and the thermal melting temperature (Tm) of the amplified product.

The required product size for RNA extracted from paraffin blocks is around a 100 bp in length, therefore the primers were designed to meet this requirement.

Primers determine which part of the RNA sequence is to be amplified by the PCR reaction and are usually 15-25bp long and designed to match specific sequences (5' and 3') flanking the segment to be amplified. The primer sequence should start and end with 1-2 purine bases, to include at least one G or C at the end of the 3' end, avoiding three G or C nucleotides in a row. The presence of the G or C acts as a CG clamp due to their strong hydrogen bonds.

For the analysis of gene transcription the primers required need to be mRNA specific and so are designed to cross an exon/exon boundary to avoid the abberant amplification of genomic DNA (where exons are interspersed with introns). Before a chosen primer is utilised, a BLAST search is performed against total sequences in GenBank specifically against the human genome to check that the primer is specific to the mRNA target (Hurteau *et al.,* 2002), and so should not readily amplify transcripts of genes other than that of interest.

Tm

The Tm of a primer represents the stability of the primer between the oligonucleotide and its complementary strand. This is determined by several different factors.

Firstly the binding between specific nucleic acid sequences is determined by interactions between complementary purine and pyrimidine bases forming A:T and G:C base pairs, with stability determined by the hydrogen bonds between these two bases.

There are two hydrogen bonds between A:T base pairs but three between G:C which makes the G:C pair more stable so requiring more energy to separate the bond. The recommended G:C content of the primers is between 40% and 60%.

In addition to the effect of the hydrogen bonds there are the effects of electrostatic forces, created by the phosphate molecules of the nucleic acid backbone and hydrophobic interactions. The proximity of the two backbones of phosphate bases means their identical ionic charged chains repel each other and so destabilise the primer. The primers can be stabilised by adding salt cations to mask the phosphate charges thereby increasing the ionic strength and the stabilisation of the double stranded sequences. Therefore primers with a higher ionic strength have a higher Tm. Hydrophobic interactions occur between staggered bases which make up the nucleic acid sequence. There are several methods to calculate the Tm of a primer, below are three different methods;

1) Wallace Rule – used for short oligonucleotides, is simple but only accurate for primers shorter than 18 bases:

   $Tm = 2 \times (A + T) + 4 \times (G + C)$

2) This is based on the %GC content and the length of the primer:

   $Tm = 81.5 + 16.6 (\log 10 [Na^+]) + 0.41 (\%GC) - (625/N)$

   N = length of the primer, Na = sodium concentration

3) Thermodynamic calculation based on entropy, enthalpy, free energy and temperature.

$$Tm = H [S + R \ln (c/4)] - 273.15°C + 16.6 \log_{10} [Na^+]$$

H = enthalpy, S = entropy (for helix formation), R = molar gas constant,

C = concentration of the primer

The best way to design a specific primer was to employ a primer design program available on the Web Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and the resultant primers were submitted to a BLAST search to check for specificity (Biosource International, 2003).

VEGF Primers

Primers were designed for the soluble isoforms $VEGF_{121}$ and $VEGF_{165}$. VEGF gene is made up of eight exons but $VEGF_{121}$ has exons six and seven removed so its primer pair was designed to cross the exon 5 and exon 8 boundary and to generate a PCR product size of 118bps. $VEGF_{165}$ has exon 6 excised so its primer pair was designed to cross the exon 5 and exon 7 boundary and to generate a product size of 123bps.

The $VEGF_C$ gene also has 8 exons. To examine $VEGF_C$, two sets of primers were determined to enable amplification separately at both the 5' and 3' end. The 5' primer pair spanned exon 1 and exon 2 to generate a PCR product size of 101bps and the 3' primer pair spanned exon 6 and exon 7 to generate a PCR product size of 92bps.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH-3) Primers

GAPDH-3 is often used as an internal house keeping gene as it should be expressed at a constant rate in cells and so is able to normalise against altered levels of specific gene products due to differences in PCR amplification, metabolism of template, altered

concentration of PCR reagents and differences in PCR cycling (Revillion *et al.*, 2000). The GAPDH-3 gene has 9 exons and its primer pair was designed to cross exon 2 and exon 3 and generate a PCR product size of 103bps. All of the primers were made by Sigma–Genosus Ltd; primer sequences are presented in Table 3.1.

Primer		Primer Sequence	No of bp	Tm (°C)
$VEGF_{121}$				
	Sense	5'-TGTGAATGCAGACCAAAGAAA-3'	21	63.2
	Anti-sense	5'-TGGTGAAGAGATATGGTTCCC-3'	20	64.1
$VEGF_{165}$				
	Sense	5'-AGGCCAGCACATAGGAGAGA-3'	20	63.9
	Anti-sense	5'-AACAATGCTTTCTCCGCTC-3'	20	63.1
$VEGF_{CI}$				
	Sense	5'-GACTCGACCTCTCGGAC-3'	18	63.8
	Anti-sense	5'-TTCATCTACACTGGACACAGACC-3'	23	62.9
$VEGF_{CII}$				
	Sense	5'-GGAAAGAAGTTCCACCACCA-3'	20	63.8
	Anti-sense	5'-TCACTATATGAAAATCCTGGCTCA-3'	24	63.8
GAPDH-3				
	Sense	5'-GAGTCAACGGATTTGGTCGT-3'	20	63.9
	Anti-sense	5'-TTGAGGTCAATGAAGGGGTC-3'	20	63.8

Table 3.1
Primer sequences for $VEGF_{121}$, $VEGF_{165}$, $VEGF_{CI}$, $VEGF_{CII}$, and GAP, with the number of base pairs (bp) in the sequence and the Tm temperature in degrees Celsius (°C).

3.3.3 RT-PCR

RT-PCR amplifies DNA sequences which are ultimately used to measure levels of RNA which can be analysed semi-quantitatively. The initial step involves the formation of complementary DNA (cDNA) from the RNA extracted from tissue samples which involves the process of reverse transcription.

Reverse Transcription (RT)

RT is required to produce cDNA which enables comparison of gene transcription between different samples. It is formed from RNA by using the viral enzyme reverse transcriptase, moloney murine leukaemia virus (MMLV). The process involves:

- Denaturation of secondary structures which RNA transcripts can exhibit
- Addition of a random hexanucleotide primer to prime reverse transcription at several points along the transcript
- Reverse transcription to produce full length cDNA.

The reverse transcription reaction has to be optimised for pH, salt concentrations and incubation temperature to give optimal conditions for the MMLV reverse transcriptase (Sigma Aldrich, Dorset, UK) to work.

Reagents for the RT reaction were prepared by cleaning the bench surface with 70% ethanol, wearing gloves, and tubes and pipettes were autoclaved. The reagents were prepared on ice at a temperature of 4°C and obtained from Sigma, UK unless stated otherwise.

For initial denaturation of secondary structures and RNA template to single strands samples were incubated at 70°C for ten minutes in a Progene Thermal Cycler, after

which the sample was primed by adding 1µl random primer (hexamers; 6 random bp). Random primers are used to initiate polymerisation by MMLV reverse transcriptase. They are short strands of DNA which are complementary to the sequence of RNA and act by priming the template strand. In addition hexamer primers have the advantage of not requiring intact poly-A tails from RNA which often fragment during paraffin fixation process. A master mix of 5x MMLV reverse transcriptase buffer, 0.1M Dithiothreitol (a reducing agent-Sigma) and 10mM Deoxynucleotide Mix (a building block for DNA synthesis, which contains dATP, dCTP, dGTP, dTTP-Sigma) was added. The mixture of RNA, primer and master mix was cooled to 42°C for 2 minutes. MMLV reverse transcriptase was then added and the temperature maintained at 42°C for 50 minutes to allow reverse transcription to take place. The action of MMLV reverse transcriptase was stopped by increasing the temperature to 70°C for 15 minutes, and the resulting cDNA was stored at -20°C. A diagram of RT-PCR is shown in Figure 3.1 and the protocol is an adaptation of the Sigma protocol shown in Table 3.2.

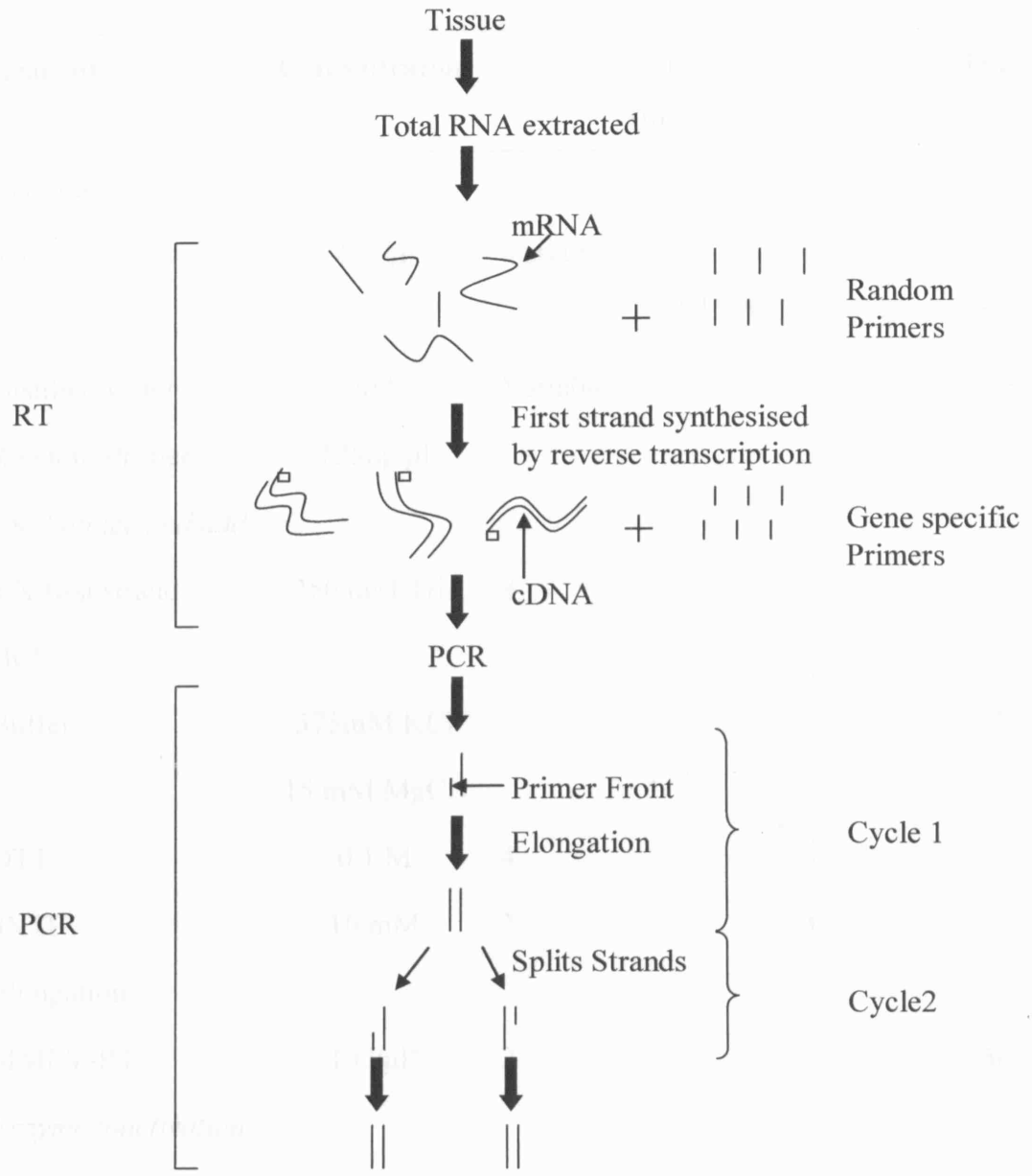


Figure 3.1
Overview of Reverse Transcription and Polymerase Chain Reaction (RT-PCR). A complete PCR involves several repeats of the PCR cycles. Modified version from Freireich & Stass (1995).

Reagent	Concentration	Volume (µl)		Temp (°C)	Time (mins)
Denaturing					
RNA	4µg/µl	Variable	Total vol 11	10	70
Distilled water	N/A	Variable			
Random Primer	125ng/µl	1			
Chill on ice and add					
5 X first strand	250 mM Tris	8			
HCl					
Buffer	375mM KCl			42	2
	15 mM $MgCl_2$				
DTT	0.1 M	4			
dNTP	10 mM	2			
Elongation					
MMLV-RT	1 U/µl[2]	2		42	50
Enzyme inactivation					
				72	15

Table 3.2
Reverse Transcription Protocol including reagents, concentrations, volumes, and length of time at a certain temperature.

Polymerase chain reaction

Polymerase chain reaction (PCR) is a technique by which a small fragment of cDNA sequence can be amplified to produce multiple copies. The technique involves repeated cycles of amplification, with each one doubling the amount of material to ultimately produce numerous copies of a single nucleic acid fragment. Amplified PCR products can then be visualised by electrophoresis.

The sequence of events is such that RNA extracted from tissue acts as a template for reverse transcription to produce cDNA. This in turn is used as a template for PCR to generate multiple copies of the desired sequence. PCR has three stages; denaturation, annealing and finally polymerisation, a schematic of PCR reaction is shown Figure 3.2.

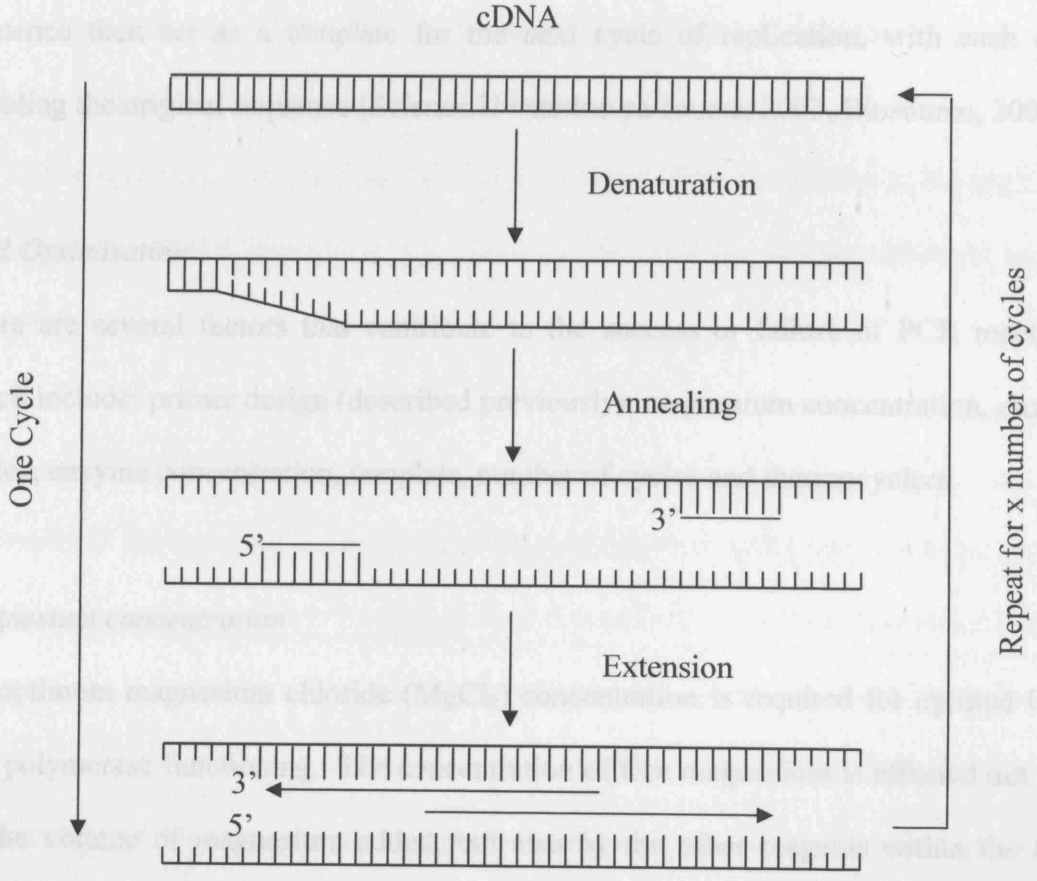


Figure 3.2
Schematic of PCR reaction, showing the denaturation, annealing and extension stages to one cycle of the PCR reaction, modified from Bradley *et al.* (1995).

Denaturation, by heating to 95°C or higher for 15 seconds to 2 minutes of the double stranded cDNA separates the two strands into single strands. Annealing involves attaching primers complementary to the sequence of cDNA which requires amplification to the single stranded cDNA sequences. Annealing is achieved by reducing the temperature to 40-60°C for 30-60 seconds. Once the primers are attached polymerisation is initiated using a thermostable DNA polymerase, e.g. Taq DNA polymerase. Taq uses the primers as a template to synthesise complementary DNA sequences. An optimal reaction temperature is required for the DNA polymerase to synthesise new DNA and for most DNA polymerases this temperature is approximately 72°C. Extension of the primer by the DNA polymerase takes approximately one to two minutes. These three stages completes one PCR cycle. The copies of the DNA sequence then act as a template for the next cycle of replication, with each cycle doubling the original sequence (Science.Uwaterloo.ca/course, 2003, Biosource, 2003).

PCR Optimisation

There are several factors that contribute to the success or failure of PCR reactions, which include; primer design (described previously), magnesium concentration, enzyme choice, enzyme concentration, template, number of cycles and thermocyclers.

Magnesium concentration

An optimum magnesium chloride ($MgCl_2$) concentration is required for optimal DNA Taq polymerase functioning. The concentration of free magnesium is affected not only by the volume of magnesium added, but also by the other reagents within the PCR reaction. Template DNA, chelating agents within the sample, dNTPs and proteins can bind to magnesium and so reduce the amount of magnesium available for Taq DNA

polymerase. However excessive magnesium decreases the enzymes function and may increase the level of non-specific amplification. Therefore, when using $MgCl_2$, from a frozen stock it was important that it was thoroughly thawed and vortexed to ensure a uniform concentration gradient.

When no PCR product is generated, as an initial problem solving step the stringency of the PCR reaction could be reduced by increasing the magnesium concentration. In contrast if multiple non-specific PCR products were generated the stringency was increased by reducing the magnesium concentration.

Enzyme Choice

Taq DNA polymerase was originally extracted from *Thermus aquaticus,* a heat loving bacterium found in the hot springs of Yellowstone National Park and is not destroyed by high temperatures. It facilitates PCR by enabling DNA production at the high PCR temperatures without denaturing Taq, limiting the need for further additions as with other enzymes.

Enzyme concentration

The optimal enzyme concentration can vary by 1 to 5 units per reaction. It was therefore important to titrate the magnesium concentration and the amount of enzyme per reaction. The use of a 'hot start', whereby the temperature of the PCR cycler is at 94°C, also helps to reduce degradation of the template and primer by the enzyme. Excess enzyme does not increase the product yield but may contribute to increase in the frequency of artefacts which are degradation artefacts generated by endogenous 5'-3'

exonuclease activity associated with Taq and can result in smearing of the PCR product on an agarose gel.

Template Considerations

The success of PCR also depends on the quantity and quality of the template cDNA. The quality of the template can be reduced by inhibitors, such as, salt, guanidine, proteases. Inhibitors can be reduced by using ethanol for the final precipitation of RNA.

For quantity, the amount of DNA template required depends on the complexity of the DNA sample and how many PCR cycles are used. Complexity is the sum of the nucleotide pairs occuring in single copy sequences and those that occur in one copy of each repeated sequence. Human genomic DNA has a complexity of 3.04×10^5 and it is ideal to start with $>10^4$ copies of the target sequence to obtain a signal within 25-30 cycles.

Number of Cycles

Amplification of DNA sequences is at a constant rate during the exponential phase of a PCR reaction (see Figure 3.3). If the number of cycles are increased, however, the amplification rate falls, and eventually the amplification rate falls to zero and the reaction plateaus. Therefore, constant amplification only occurs during the exponential phase so the number of cycles to a PCR reaction has to be altered to fall within this phase. Graphs of the PCR reaction rate is shown in Figure 3.3. The PCR cycle protocol used is shown in Figure 3.3.

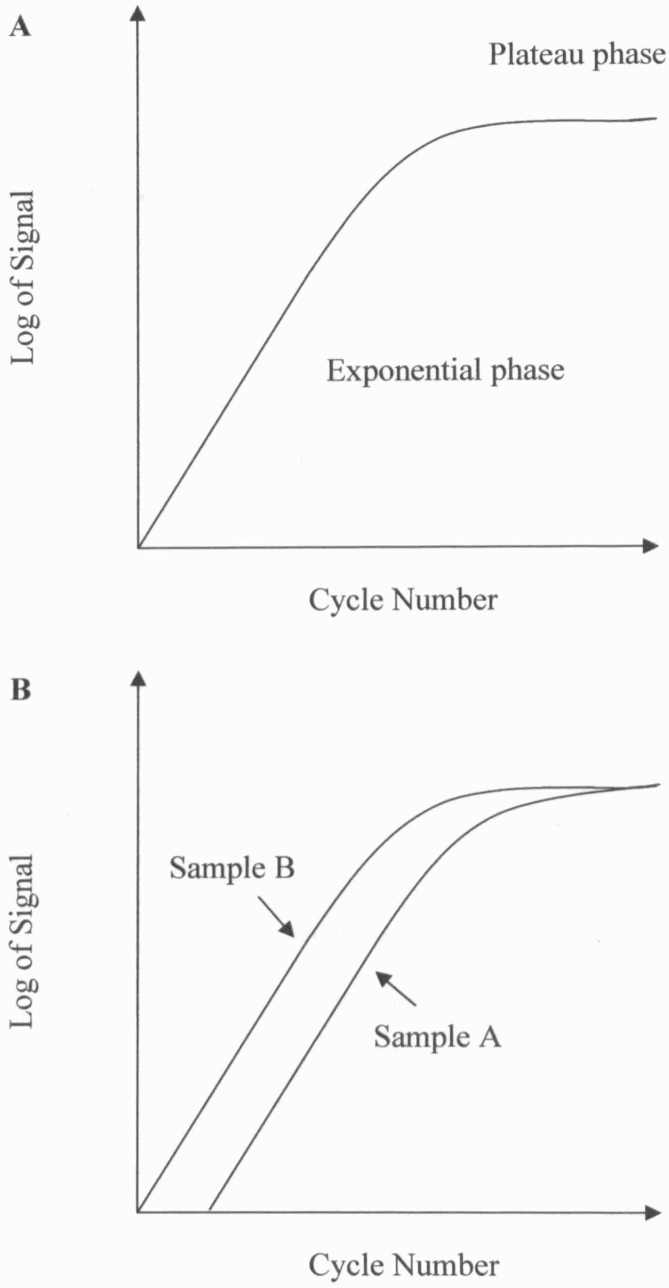


Figure 3.3
PCR reaction. **A** - demonstrating the difference in rate of reaction with different number of cycles. **B** - demonstrates the different rate of PCR reaction with 2 samples if the rate is not in the exponential phase. Modified from Bradley *et al.* (1995).

Step	PCR Cycles		Time (sec)	Temp (°C)
1	10			
		Denaturation	45	94
		Annealing	45	50
		Extension	45	72
2	10			
		Denaturation	45	94
		Annealing	45	55
		Extension	45	72
3	20			
		Denaturation	45	94
		Annealing	45	58
		Extension	45	72

Table 3.3
PCR Cycling Protocol

A table of how to solve PCR problems is shown in Table 3.4.

PCR Problem	Suggestions
No Band	Decrease stringency by - decreasing annealing temperature - & / or increasing [Mg^{2+}]
	Increase number of cycles
	Redesign primers
	No target DNA is present
Too Many Bands	Increase stringency by - increasing annealing temperature - & / or decreasing [Mg^{2+}]
	Decrease cycle number
	Perform a hot start PCR
	Decrease primer & / or template concentration
	Verify primer banding site is unique
Wrong Size Band	Verify primer sequence is correct
	Increase annealing temperature
	Use hot start PCR
Primer Dimers	Set up reactions on ice and perform a hot start PCR
	Lower the primer concentration (try 50-100nM)
Band in –ve control lane	Treat RNA sample with DNA free DNAse treatment and removal reagents

Table 3.4
PCR trouble shooting summary.

The reagents and protocol used for the PCR are shown in Table 3.5 and 3.6 respectively.

Reagents	**Stock Conc. (mM)**	**Conc. used In PCR**
$MgCl_2$	25	4 mM
Red Taq™		1 unit/µl
Deoxynucleotides		
TTP, ATP, GTP, CTP	100	10 mM
10x PCR Buffer		
Trizma-HCl	100 (pH 8.3 at 25 °C)	10 mM
KCl	500	50 mM

Table 3.5
Reagents used in polymerase chain reaction from Sigma, UK, showing stock concentration and the actual concentration used.

Reagent	Concentration	Volume (µl)	Temp (°C)	Time (mins)
Denaturation				
cDNA	600µl	3		
Magnesium Chloride	4mM	4		
10x RED Taq PCR buffer		4	94	4
Primer	100ng/µl	1		
H_2O		To make up total vol to 45µl		
Taq mixture added				
H_2O		4		
10x RED Taq PCR buffer		0.5		
Taq DNA Polymerase	1unit/µl	0.5		

Table 3.6
PCR Protocol including reagents, volumes, concentrations, and length of time at a certain temperature.

Thermocyclers

There are numerous different thermocyclers and the same PCR reaction on different PCR thermocyclers may give a different result. Different tubes can also affect the PCR reaction, due to different shapes and wall thickness which leads to imperfect contact between the tube and the metal block of the thermocycler. The end result is uneven heat transfer between the thermocycler and PCR tube leading to variations in PCR amplification (Biosource, 2003).

3.3.4 Analysis of gene transcription

Semi-quantitative Analysis

Semi-quantitative analysis of RT PCR was used to determine whether one transcript was expressed higher than another. The method is based on using an internal control included in the same PCR reaction as the gene transcript of interest. The internal control is a house keeping gene such as, glyeraldehyde 3 phosphate dehydrogenase (GAPDH 3; GAP-3), which should be transcribed at a constant level. Normalisation of experimental results by GAPDH 3, controls variations between samples which have occurred during the PCR reaction. The PCR reaction was validated using a positive and negative control.

PCR products were separated by agarose gel electrophoresis and were analysed for relative gene expression and in these reactions they were analysed semi-quantitatively. (Science.uwaterloo.ca/course notes, 2003).

Agarose Gel Electrophoresis

Agarose gel electrophoresis separates DNA fragments according to size. Agarose is made up of a chain of sugar molecules extracted from seaweed. A powder, at room temperature, agarose dissolves when heated to boiling point in water. When cooling agarose it polymerises and the sugar molecules cross link to form a semi solid matrix in which negatively charged DNA fragments can move when an electric current is applied.

Different concentrations of agarose within a gel separates a wide range of fragment sizes ranging from ~500bp to 20,000bp. If the agarose concentration is reduced to 0.5% then less than 2000 bp is challenged by the agarose matrix and the fragments migrate at an equal distance within the same time period and separation does not occur. A far better separation of small fragments (100bp) is obtained with 2% agarose because these fragments are challenged by high level of agarose.

A 2% agarose gel (RNA grade; BDH, UK) was prepared by adding 3mg of agarose to 150ml of TBE (Sigma UK, prepared from stock of 10x and diluted with deionised water); T - Tris which maintains a constant pH, B - Boric which provides the proper ion concentration for the buffer, E - EDTA which chelates divalent cations e.g. magnesium. The agarose is dissolved in TBE by boiling in a 800W microwave for 2 minutes, and after the agarose had cooled slightly 10µl of ethidium bromide (Sigma, UK) was added. The gel is then poured into a gel tray and 'combs' are applied to form the wells used to load the PCR product and left to stand for 30 minutes to allow the gel to set. PCR products are the same density as the buffer so if it is loaded in to the wells it would float, which is prevented by adding a loading buffer which contains 30% glycerol in water and 0.25% bromophenol blue to increase the density of the PCR product. The

bromophenol blue in the buffer allows localisation of the PCR products during electrophoresis. The gel tray was sealed at either end before the liquid agarose gel was poured into it. The more base pairs of DNA in a PCR product the more ethidium bromide there is wedged into the grooves of DNA and the brighter the band on uv translumination.

Once the samples were loaded into the wells of a gel an electric current is applied from the positive to the negative electrode, so that the negatively charged DNA moves towards the positive charge. A 60mA current at 120 mV was applied to the electrophoresis gel for 30 minutes and the gels visualised under uv light and a Polaroid photograph is taken (Molecular Biology CyberLab, 2003).

Scanning Densitometry

Polaroid photographs of the resulting gel electrophoresis was scanned onto the computer for analysis using Epson GT-9500 scanning densitometer (Epsom, Japan). The image was then exported to the image analysis programme (Labworks^{TM} Image acquisition and analysis software, ultraviolet products, Cambridge UK). The Integrated Optical Density (IOD) of each band was estimated and the level of gene transcription was measured as a ratio of the IOD of the gene compared to GAP-3.

The levels of gene transcription were analysed for gender, age, site of tumour, Duke's stage, MSI and its mutated markers.

3.4 Histology and Immunohistochemistry

3.4.1 Haematoxylin and Eosin

Haematoxylin and Eosin is a technique which is used to stain the nuclei of cells based on the theory of attraction of oppositely charged tissue and dye molecules. Haematoxylin stains the cell nuclei blue/black whereas eosin stains the cell cytoplasm and most connective tissue fibres in varying shades and intensities of pink, orange and red.

Mayer's Haematoxylin

Mayer's haematoxylin, is chemically oxidised by sodium iodate and it is an alum haematoxylin. It is used as a regressive stain which is useful when a nuclear counter stain is needed to emphasise a cytoplasmic component. In regressive staining the tissue is overstained and then differentiated in an aqueous or alcoholic hydrochloric acid solution to achieve the required staining. The colour shifts from blue/purple to salmon pink/red and after differentiation the sections are rinsed in dilute alkaline blueing solution to re-establish the blue dye.

Eosin

Eosin is widely used as a counterstain with maximum uptake by the basic cytoplasmic proteins when used at pH 4.6 to 5.0. At the appropriate pH eosin produces three shades of pink stain, with different intensities of dye on erythrocytes, collagen and the cytoplasm of muscle or epithelial cells. The different shades of pink result from eosin molecules causing rearrangement of chemical bonds which produce slightly different colours of the dye (Histological staining, 2003).

Prior to staining of sections the samples are dewaxed by xylene and then dehydrated by ethanol. Once staining is completed this process is reversed prior to mounting in 1,3-diethyl-8-phenylxanthine (DPX).

Haematoxylin and Eosin Protocol

The tumour blocks was cut into 5 micron sections and left to air dry for at least two hours. The sections were then placed in a 60°C oven for 20 minutes, and then the protocol shown in Table 3.7 and 3.8, is undertaken in a fume cupboard due to the toxic nature of the chemicals.

Procedure	**Time (minutes)**
Dewax section in xylene	3
Dewax section in second xylene	3
100% ethanol	1
70% ethanol	1
Place slides under running tap water	1
Stain slides in Harris' Haematoxylin (regressive stain)	5
Wash slides in running tap water	3
Differentiate in 1% acid alcohol	6 sec
Wash in Scott's tap water until sections are blue	~ 5

Table 3.7
Haematoxylin and Eosin Protocol.

Procedure	Time (minutes)
Wash briefly in tap water	<1
Stain slides in 1% eosin	5
Wash in running tap water	0.5-1
Place slide in 70% ethanol	15 sec
100% ethanol	30 sec
100% ethanol	30 sec
Xylene	1
Xylene	1
Mount with DPX	
Cover with coverslip and allow to air dry	

Table 3.8
Haematoxylin and Eosin Protocol (cont).

3.4.2 Immunohistochemistry

This technique is used to identify the expression of proteins in tissue by using an antigen-antibody interactions, where proteins act as an antigen. An appropriate labelling system is required by either a direct or secondary method.

Antibody Selection

Ig G is one of the most abundant immunoglobulins and it is used in immunohistochemistry for three reasons;

- There are two sites which are the ends of Fab segments capable of binding to an antigenic determinant. This gives the antibody its bivalent characteristics.
- Part of the Ig G molecule, Fc fragment is common to all antibodies of the same species and is not involved in combining with antigens.
- Ig G may have several different antigenic sites that bind to more than one molecule of an anti-antibody.

The Ig G molecule is large with a molecular weight of 150 000D which allows other compounds, such as enzymes or flourochromes to conjugate to Ig G amino amide side chains. This process is known as labelling and the ability of an antibody to bind is not affected by labelling provideed the labelling molecule does not sterically obstruct the distal parts of the Fab segments. The labelling agents molecules can bind to the different parts of the antibody randomly and so can bind to the distal parts of the Fab segments and block the binding sites. This means that the potency of the antibody is reduced by addition of a label (Boenisch, 2003).

Polyclonal Antibodies

These antibodies are produced by different cells and are immunologically dissimilar. They react to different epitopes on the antigen against which they are raised. The animal used to produce polyclonal antibodies is rabbit, goat, pig or sheep, rabbit being most commonly used as human antibodies to rabbit proteins are rarer than to other animals and rabbit antibodies precipitate human proteins over a wider range of antigen or antibody excess.

Monoclonal Antibodies

Monoclonal antibodies are the product of an individual clone of plasma cells. Antibodies from a given clone are immunochemically identical and react with a specific epitope on the antigen against which they are raised.

Monoclonal antibodies have an advantage over polyclonal antibodies by having high homogeneity, absence of non specific antibodies, and no batch to batch variability and ease of characterisation.

The disadvantage of monoclonal antibodies is they sometimes do not bind to antigens which have been formalin fixed, whereas, polyclonal antibodies do. The specificity of a monoclonal antibody is one of its benefits but if lost the antibody can bind to two or more different antigens. The cross reactivity of a polyclonal antibody but not monoclonal antibodies can easily be eliminated by affinity absorption of the antiserum (antiserum that has been absorbed with immunoglobulins from the species under investigation). The function of a monoclonal antibody is also more dependant on factors such as pH of the solute and its optimum temperature (Boenisch, 2003).

Antibody Affinity

A specific antibody can have both intrinsic and functional affinity. The intrinsic affinity is determined by the variable domains in the heavy chains and this determines the specificity of the antibody. However a greater specificity does not equate with a greater affinity of the antibody as the affinity is affected by the ionic interactions, hydrogen bonding, and van der Waals forces between the antibody and antigen.

The functional affinity of an antibody is the time required to reach equilibrium with the tissue antigen. The antibody which reaches a plateau of maximum staining intensity first has the highest functional affinity.

Antigen-antibody reactions are reversible and the simple immune complexes formed on the tissue may dissociate during the washing cycles used in immunohistochemistry. The ease and degree of dissociation varies with antibodies but the risk of this occurring is reduced by a low salt concentration and a low temperature. Then high affinity antibodies are less likely to dissociate than low affinity antibodies during washing. Polyclonal antibodies are a mixture of high and low affinity and so dissociation is unlikely to occur. In contrast monoclonal antibodies are all of the same affinity so if the antibody has a low affinity loss of staining is likely to occur due to dissociation and therefore it is necessary to choose antibodies with a high affinity (Boenisch, 2003).

Antibody Reaction Rates

The reaction rates are affected by tissue fixation, antibody concentration, ambient temperature and incubation time of the primary antibody. Over fixation of tissue may make it difficult for antibodies to penetrate the tissue and the reaction rate is decreased.

In order to potentiate reaching an equilibrium between bound antigen and free antibody more dilute antibodies and longer incubation times have been used rather than high concentrations and shorter incubation times as this increases the non specific background which can affect the results of immunohistochemistry.

Antibody Titre

The optimal antibody titre is defined as the highest dilution of an antiserum that results in maximum specific staining with least amount of background under specific test conditions. The highest dilution is determined by the absolute amount of specific antibodies present and it is also affected by the intrinsic affinity of an antibody. Titres may vary from 1:100 to 1:2000 for polyclonal antibodies and 1:10 to 1:1000 for monoclonal antibodies.

Antibody Dilution

The antibody dilution required for immunohistochemistry is as recommended by the manufacturers, otherwise it is best to start with a fixed incubation time and then carry out a series of experimental dilutions. A 100 to 200µl of antibody usually covers a section adequately and the optimal concentration is shown by a peak in staining intensity with a minimal back ground.

Monoclonal antibodies are more sensitive to pH and ions within the buffer. The recommended pH is 6.0 and 8.6 and phosphate buffered saline is widely used as a dilutent for primary antibodies which suppress the reactivity of monoclonal antibodies.

Antibody Incubation

There is an inverse relationship between incubation time and antibody titre, the higher the antibody titre the shorter the incubation time required for optimal results. Incubation times are mostly 10-30 minutes but can be up to 48 hours. When a long incubation is required, temperature is maintained at 4°C and the sections are not allowed to dry out during incubation and by using a humidity chamber.

CD34 endothelial cell marker QBEnd10 (Skybio, UK), monoclonal antibody is used to detect blood vessels so that blood vessel density was calculated. It is a mouse monoclonal immunoglobulin G, sub-class 1 and the stock concentration was 50ug/mL. A dilution of 1/50 was used. This antibody is selectively expressed on human lymphoid and myeloid haemopoietic progenitor cells and it stains positively for vascular and lymphatic tumours.

$VEGF_C$ polyclonal antibody IgG (RD Systems, UK) is the antibody used to detect protein C expression. It is produced in goats immunised with purified, E. coli-derived recombinant $fhVEGF_C$ peptide corresponding to amino acid residues Glu 104 to Ala 330. The antibody was reconstituted in 1ml PBS and the concentration of the stock was 0.1 mg/ml. A concentration of 8µg/ml was used for immunohistochemistry.

Enzymes

Enzymes are chromogens used to convert colourless chromogens into coloured end products and the activity is dependant upon several factors, for example, enzyme and substrate concentrations, pH, salt concentration of the buffer, temperature and light.

Many enzymes also require metal ions Mg^{2+}, Mn^{2+} and Zn^{2+}, which act as electrophilic agents.

Selection of an enzyme

Selecting the appropriate enzyme depends on a number of criteria;

- The enzyme should be available in highly purified form and relatively inexpensive.
- Conjugation or noncovalent binding although may diminish, should not abolish enzyme activity.
- The bound enzyme should be stable in solution.
- Endogenous enzyme activity should interfere minimally with specific antigen related staining.
- The products of the enzymic reactions should be readily detectable and stable.

Horseradish Peroxidase (HRP)

This enzyme is isolated from the horseradish plant and it has an iron containing heme group (hematin) as its active site and in solution is coloured brown. The hematin of HRP forms a complex with hydrogen peroxide and it then causes it to decompose resulting in water and atomic oxygen. HRP oxidises several substances, for example polyphenols and nitrates and two step conjugation of HRP results in more efficient labelling of the antibody and no polymerisation. This involves HRP reacting with bifunctional reagents first and then activated HRP is admixed with the antibody. HRP activity in the presence of an electron donor first results in the formation of an enzyme substrate complex and then in the oxidation of the electron donor. The electron donor provides the driving force in the continuing catalysis of H_2O_2, while its absence

effectively stops the reaction. There are several electron donors when oxidised become coloured products and are chromogens.

3,3'-Diaminobenzidine (DAB)

This chromogen produces a brown end product which is highly soluble in alcohol and other organic solvents. Oxidation of DAB causes polymerisation which increases its staining intensity and electron density (Boenisch, 2003).

Antigen Retrieval

Fixation of tissue by formalin can cause loss of immunoreactivity of many antigens. The differences in fixation protocols with for example, pH and exposure to chromogens influence the outcome of staining. These differences can cause cross linkage between tissue proteins and their antigenic sites which make it difficult to predict the outcome and it can result in partial or complete loss of immunoreactivity by the antigen and/or can mask it.

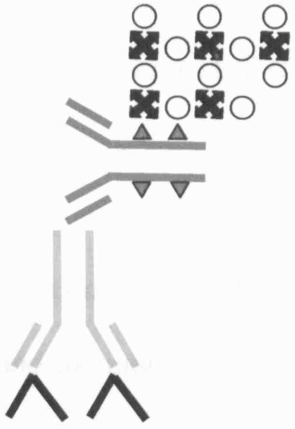
The process of antigen retrieval is used to improve the immunoreactivity by unmasking and retrieving antigen sites and a number of techniques have been introduced, the two principal methods are either by tryptic digestion or by heat and citrate buffer, which was used in this study. The citrate buffer was preheated before the slides were exposed to heat by boiling in an open pressure cooker. The solution and slides were exposed to pressurised heat for a period from a few minutes to an hour. The length of time is determined by the length of formalin fixation and the antibiotics used and it is followed by slowly running cold water into the cooker until the slides were cooled which usually takes 20 to 30 minutes (Boenisch, 2003).

Antigen blocking

Antigen blocking is a process which reduces the non specific binding of protein molecules and involves the treatment of the section with a solution of protein to block non specific binding sites so that the antibody reagents adhere only to antigens to which they have a high affinity. The reagent solution used to achieve this is a protein which is the normal serum from which the secondary antiserum was raised. (Kiernan, 2000).

Staining methods

There are different methods of staining antibody and antigen reactions which can be direct or indirect. The indirect method has the advantage of amplifying the signal so more enzymes are attached per target site and is more sensitive than the direct method. The soluble enzyme immune complex technique uses a preformed soluble enzyme anti enzyme complex which is obtained by adding excess enzyme to its antibody and any precipitate is removed. This technique uses unconjugated primary antibody, secondary antibody, soluble enzyme anti enzyme complex and substrate solution. Secondary antibody is added in excess so one of its two Fab sites binds to the primary antibody and thus leaves other sites available to bind to the antibody of the enzyme immune complex.



Streptavidin biotin enzyme complex

Biotinylated Secondary Antibody

Primary Antibody

Antigen

(Adapted from Boenisch, 2003)

The results of this technique are more sensitive due to greater number of enzyme molecules localised per antigenic site. This technique is based on the high affinity of streptavidin (*Streptomy cesavidinii*) and avidin (chicken) have for biotin, which are used as the soluble enzyme anti enzyme complex, as they both possess four binding sites for biotin. An outline of this method is primary antibody, biotinylated secondary antibody followed either by (strept) avidin-biotin enzyme complex (ABC) technique or by enzyme labelled streptavidin and the reaction ends with substrate chromogen. Horseradish peroxidase and alkaline phophatase are the most commonly used enzyme label (Boenisch, 2003).

Counter staining

Various stains can be used for counterstaining and this is used to identify the nuclei of cells. Mayer's haematoxylin can be used and its mechanism of action has been described previously (Section 3.4.1).

Controls

Controls are required to validate the staining results and this can be a negative, positive or an internal control. For the analysis of blood vessel density and $VEGF_C$ protein expression negative and tissue controls were used (Boenisch, 2003).

Background

Background staining is the most common problem with immunohistochemistry. There are several causes of background staining.

Hydrophobic interactions

Hydrophobic interactions occur due to van der Waals forces which form between macromolecules when their surface tensions are lower than water. Hydrophobicity is one of the forces which contributes stability to the formed immune complexes and can exist between different protein molecules. Proteins are made more hydrophobic by fixation and the increased hydrophobicity results from cross linking. The extent of the cross linkage is a function of time, temperature and pH during fixation. Changes in these factors result in variable hydrophobicity and so fixation procedures need to be maintained and controlled. Most background staining is a result of hydrophobic interactions of connective tissue, squamous epithelium and adipocytes if they are not completely removed by xylene.

Immunoglobulins are particularly hydrophobic and storage of immunoglobulins increases their hydrophobicity and leads to aggregation and polymerisation which leads to a diminution or loss of immune reactivity. The hydrophobic interaction between cross linked proteins and antibody can be minimised by adherence to optimal fixing conditions.

Hydrophobic binding between monoclonal Ig G and tissue proteins is reduced by adding a detergent (e.g. Tween 20) or ethylene glycol to the diluent or by raising the pH of the diluent used for polyclonal antibodies.

The most common method used to reduce the background is to block the protein or add antibody diluent.

Ionic Interactions

Ionic interactions contribute to non specific background, for example, negatively charged sites on endothelium and collagen interact with cationic conjugates composed of rabbit Fab fragments and horseradish peroxidase type VI. These ionic interactions can be reduced by using diluent buffers of higher ionic strength. Most diffuse background is due to a combination of ionic and hydrophobic interactions and remedies for one type of interaction may aggravate another.

Endogenous Enzyme Interactions

Incubating with 3% hydrogen peroxide for 5 to 10 minutes is used to suppress endogenous enzyme interactions.

Natural and Contaminating Antibodies

Natural antibodies are present in the antiserum as a result of prior environmental antigenic stimulation, however most natural antibodies are nonprecipitating and occur at relatively low concentrations. These antibodies are usually rendered non reactive on tissue if antiserum is used at a sufficiently high dilution or by shortening the incubation time period.

Contaminating antibodies are present in low concentration and will not detract from the immunohistochemical specificity of the antiserum if they are diluted sufficiently. However contaminating antibodies can cause non specific staining of the tissue. The problems of natural and contaminating antibodies do not occur with monoclonal antibodies.

Antigen Diffusion

Background staining can occur if the tissue marker to be stained has moved from its sites of synthesis or storage into the surrounding tissue. For example, the tissue marker may be present in high concentration in blood plasma and may have perfused the tissue before it is fixed. This emphasises the importance of fixing the tissue promptly.

Cross Reactivity

Cross reactivity occurs when several epitopes of the target tissue antigen are shared with other proteins. Careful cloning of monoclonal antibodies eliminates this type of background staining.

Miscellaneous Sources

Diffuse staining of all or most tissue elements may be caused by physical injury to tissue by tissue drying out prior to fixation or by incomplete penetration of the fixative into the tissue. Care should be taken that sections are mounted in water baths that are free from bacterial or yeast contamination (Boenisch, 2003).

The reagents and protocol used for the immunohistochemistry are shown in Tables 3.9, 3.10 and 3.11.

Reagent	Make	pH	Protocol
Phosphate buffered saline	Sigma, UK	7.4	2 tablets of PBS + 400mls sterile H_2O
Citrate buffer	Sigma, UK	6.0	29.4 g sodium citrate + 10l H_2O + ~ 22mls conc. HCl to make pH 6.0
3% Hydrogen peroxide	BDH, UK		1ml 30% H_2O_2 to 9mls sterile H_2O
Blocking serum	Vector Labs Ltd		3 drops of stock normal serum to 10mls PBS
Biotinylated antibody	Vector Labs Ltd		1 drop of stock antibody to 10mls PBS
Vectastatin ABC reagent	Vector Labs Ltd		2 drops of reagent A to 10mls PBS in ABC mixing bottle 2 drops of reagent B to mixing bottle
DAB	Sigma, UK		1 DAB tablet + 1 urea hydrogen peroxide tablet to 15mls sterile H_2O

Table 3.9
Immunohistochemistry reagents used, including make, pH and protocol.

Reagents	Time (min)
Xylene	2 x 2
100 % ethanol	2 x 2
70 % ethanol	2 x 2
Wash in tap water	5
Boil 3l citrate buffer when boiling add sections	CD 34 for 2
Put on lid of pressure cooker then pressurise	$VEGF_C$ for 3
Dry excess fluid and mark with hydrophobic pen	
Incubate with 3% hydrogen peroxide	10
Wash with buffer	5
Incubate with blocking antibody	CD34 for 0
	$VEGF_C$ for 20
Blot off excess blocking antibody	
Incubate with primary antibodies	CD 34 for 60
	$VEGF_C$ for 60
Wash with buffer	5

Table 3.10
Immunohistochemistry protocol. Three micron sections were cut from wax blocks, allowed to drain for two hours and then incubated overnight at 60°C. The above protocol was then used, which includes reagents and length of time used.

Reagents	Time (min)
Incubate with secondary antibody	30
Wash with buffer	5
Incubate with ABC Reagent	30
Wash with buffer	5
Incubate with DAB	CD 34 for 30
	$VEGF_C$ for 4
Wash with tap water	5
Counter stain with Mayers Haematoxylin	2
Wash with tap water until sections are blue	3
Dip in and out of acid alcohol	
Wash with tap water until sections are blue	5
70% ethanol	2 x 2
100% ethanol	2 x 2
Xylene	2 x 2
Mount in DPX	

Table 3.11
Protocol for immunohistochemistry (cont).

3.4.3 Analysis of protein expression and blood vessel density

Protein Expression of $VEGF_C$

Sections were identified as positive for $VEGF_C$ protein when there was staining in the cytoplasm. Negative sections were those which had no staining or unclear weak reactions (Nouguchi et al., 2002). The percentage of staining of cytoplasm was a measured protein expression.

Blood vessel density

Initial scanning of the tumour section was at 10x magnification and the tumour section and adjacent stromal tissue (within a diameter of one field at x200 magnification), was used to identify the areas with maximum number of microvessels (hotspot). The hot spot was analysed at a higher magnification (x200-250) using an eyepiece graticule containing 25 randomly positioned dots (Chalkley point). The eye piece was rotated so that the maximum number of points was on or within the vessels of the vascular hot spot. The points on the graticule were counted instead of microvessels. The blood vessel density was counted by two blinded independent investigators who were blinded to the tumour characteristics. A blind recount was carried out if there was greater than 10% difference in the two counts (Vermeulen *et al.*, 1996).

The levels of protein expression were analysed for site of tumour, Duke's stage, MSI and MSI nucleotide markers that were mutated.

3.5. Statistics

The results were analysed using descriptive statistics. The ages were represented as median values with ranges in parenthesis and comparisons between group ages were made by Mann-Whitney U test. Chi-squared test was used to compare MSI status with Duke's stage, gender and site and Duke's stage with gender or site. The levels of gene transcription, BVD and percentage of cells staining for $VEGF_C$ were shown graphically and the mean value was indicated and the s.e.m. was shown in parenthesis. These results were analysed for uniformity and skewness and by one-sample Kolmogorov-Smirnov test for normality. Comparisons between groups were then made by one-way analysis of variance (ANOVA) with Bonferroni's correction, or unpaired Student's t test, to identify differences between individual group means. Correlation between factors was analysed by Pearson's Bivariate correlation analysis. Statistical significance was accepted at $p<0.05$.

CHAPTER 4

MSI IN SPORADIC COLORECTAL CANCER

CHAPTER 4

4.1 Background

The incidence of CRC increases with age, with rectal and colon cancer developing mostly between the ages of 60 and 69 years (Goligher, 1941, 1984, Chi *et al.*, 1994). The majority of CRC develop in the left colon and rectum (Dukes, 1940, Goligher, 1941). CRC can be classified according to Duke's classification but in addition it can be classified by the degree of unstable MSI markers. MSI-H tumours are those with ≥30-40% unstable markers, MSI-L when <30-40% markers are unstable and MSS when no markers are unstable (Boland *et al.*, 1998). MSI-H tumours have been shown in previous studies to have different phenotype compared to both MSI-L and MSS tumours. MSI-H tumours are predominantly found in the right colon (Chao *et al.*, 2000, Gryfe *et al.*, 2001), in female (Ward *et al.*, 2001) and elderly patients (>70 years; Chao *et al.*, 2000). In addition, they have a lower incidence of lymphatic and distant spread and a better prognosis when stage matched with MSS tumours (Gryfe *et al.*, 2001, Wright *et al.*, 2000). Prior to initiating any experimental analysis, the sample group chosen for study in this thesis was first analysed to verify that it was representative of the overall pattern of CRC in the population and of MSI in CRC. The MSI phenotype was analysed for gender and age of the patient and site and Duke's stage of the tumour.

4.2 Aim

To examine the occurrence of MSI in the study samples of CRC tumours, and in relation to gender, age, location and Duke's stage of the tumour.

4.3 Methods

A retrospective analysis of sixty-seven (67) CRC from the Thames cancer registry, in which the MSI status had been previously determined, was undertaken. The patient age and gender, and location and Duke's stage of each tumour was recorded. The MSI status was then investigated to see if there was any relationship with the above demographic factors, to determine whether the samples chosen for study were representative of CRC, and of MSI in CRC (see Chapter 3.1 and 3.2).

Eight (8) normal samples of colon were also used for comparison to the tumour samples. These colon samples where taken from patients who had undergone resection from a different part of the bowel. The demongraphics of each patient were documented.

4.4 Results

Patient demographics

There were 35 (52%) males and 32 (48%) females. The median age was 72 years with a range from 38 to 100 years; 28 (42%) patients were less than 70 years old and 39 (58%) were older than 70 years old (see Table 4.1). The raw data is presented in Appendix I.

Tumour characteristics

Of the 67 samples, 28 (42%) were located in the right colon and 39 (58%) located in the left colon. There were 6 (9%) Duke's A, 32 (48%) Duke's B, 24 (36%) Duke's C and 5 (7%) Duke's D. The MSI status was distribution was 12 (18%) MSI-H, 18 (27%) MSI-L and 37 (55%) MSS (see Table 4.1).

Demographics	Number of patients (% of total)
Gender	
Female	32 (48%)
Male	35 (52%)
Age	
<70 years	28 (42%)
>70 years	39 (58%)
Site	
Right	28 (42%)
Left	39 (58%)
Duke's	
A	6 (9%)
B	32 (48%)
C	24 (36%)
D	5 (7%)

Table 4.1
Distribution of gender and age of patients and location and Duke's stage of tumours. The numbers represent total numbers with percentages of total numbers are shown in parenthesis.

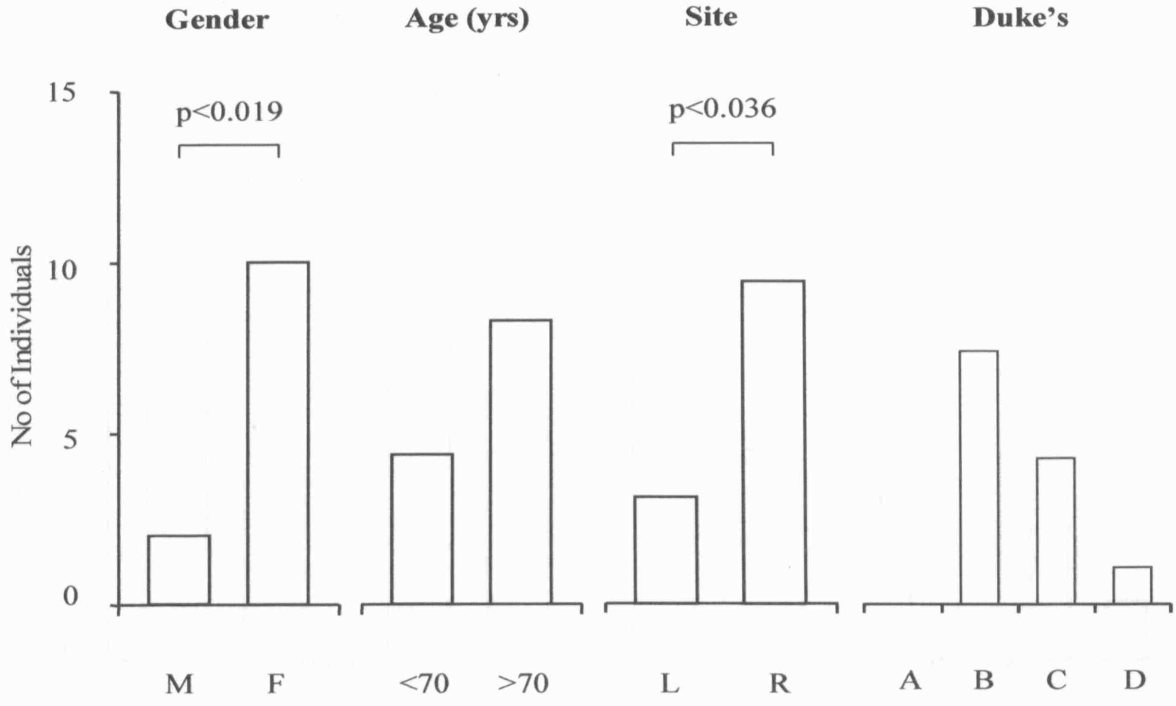
Of the eight normal colon specimens, 5 (63%) were from males and 3 (27%) females. The median age was 69 years with a range of 45 to 87 years. 7 (88%) of colon samples were taken from the left colon and 1 (12%) from the right colon. The tumours which were resected from these patients were 1 (12.5%) tubular adenoma, 2 (25%) Duke's A, 4 (50%) Duke's B and 1 (12.5%) Duke's C (see Table 4.2). The raw data is presented in Appendix I.

Demographics	**Number of patients (% of total)**
Gender	
Female	3 (27%)
Male	5 (63%)
Site	
Right	1 (12%)
Left	7 (88%)
Duke's	
A	2 (25)
B	4 (50%)
C	1 (12.5)
D	0 (0%)
Tubular Adenoma	1 (12.5%)

Table 4.2
Distribution of gender of patients, and site of normal colon samples. Duke's stage of tumours resected from these patients. The total numbers are shown with percentages of total numbers shown in parenthesis.

MSI-H tumours

MSI-H tumours were predominantly found in females (Figure 4.1, $p<0.019$, Pearson Chi-square test). The majority, 8 (67%), were older than 70 years with 4 (33%) less than 70 years old (Figure 4.1, $p>0.05$, Pearson Chi-square test). Nine (9; 75%) of the MSI-H tumours were located in the right colon and 3 (25%) were in the left colon (Figure 4.1, $p<0.036$, Pearson Chi-squared test). MSI-H and Duke's stage, 0 Duke's A, 7 (58%) B, 4 (33%) C, 1 (8%) D (Figure 4.1, $p>0.05$, Pearson Chi-squared test).

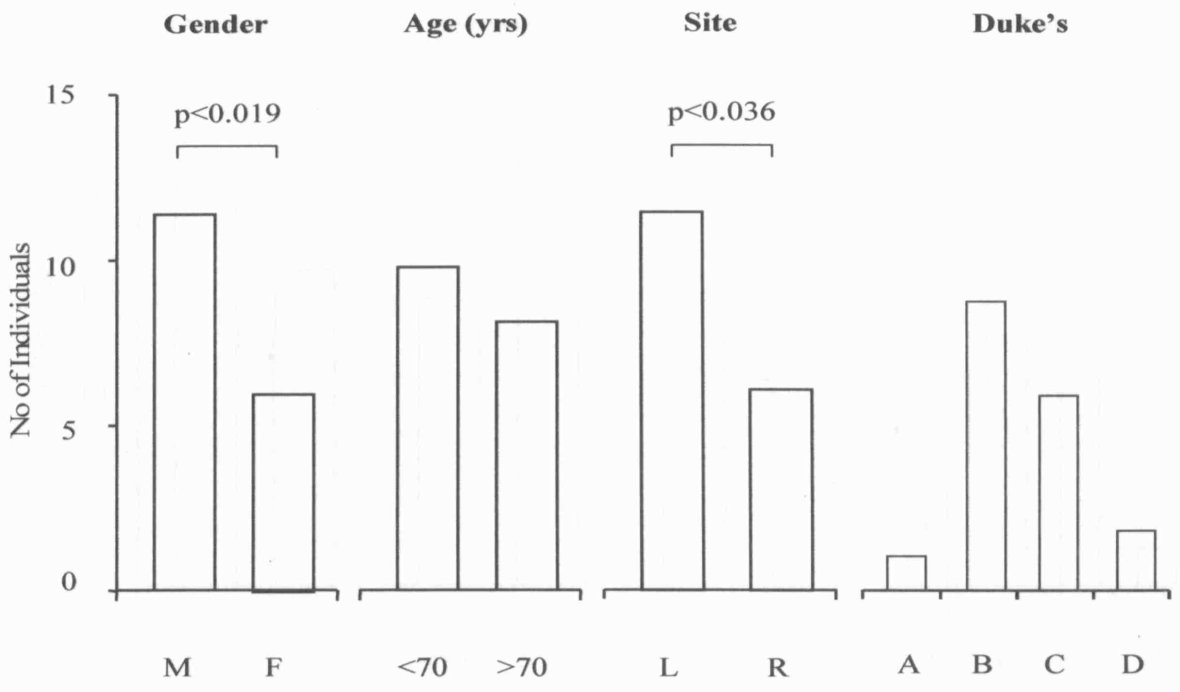


Gender		Age (years)		Site		Duke's Stage			
Male	Female	< 70	> 70	Left	Right	A	B	C	D
2	10	4	8	3	9	0	7	4	1
(17%)	(83%)	(33%)	(67%)	(25%)	(75%)	(0%)	(58%)	(33%)	(8%)

Figure 4.1
Distribution of gender, age, site of tumour and Duke's stage of MSI-H tumours. The numbers represent total numbers with percentages of group are shown in parenthesis.

MSI-L tumours

MSI-L tumours, 6 (33%) were females and 12 (67%) males patients (Figure 4.2, $p<0.019$, Pearson Chi-square test). Ten (10; 56%) patients were less than 70 years old and 8 (44%) were older than 70 years (Figure 4.2, $p>0.05$, Pearson Chi-square test). Six (6; 33%) of the MSI-L tumours were located in the right colon and 12 (67%) were in the left (Figure 4.2, $p<0.036$, Pearson Chi-squared test). MSI-L and Duke's stage, 1 (6%) Duke's A, 9 (50%) B, 6 (33%) C, 2 (11%) D (Figure 4.2, $p>0.05$, Pearson Chi-squared test).

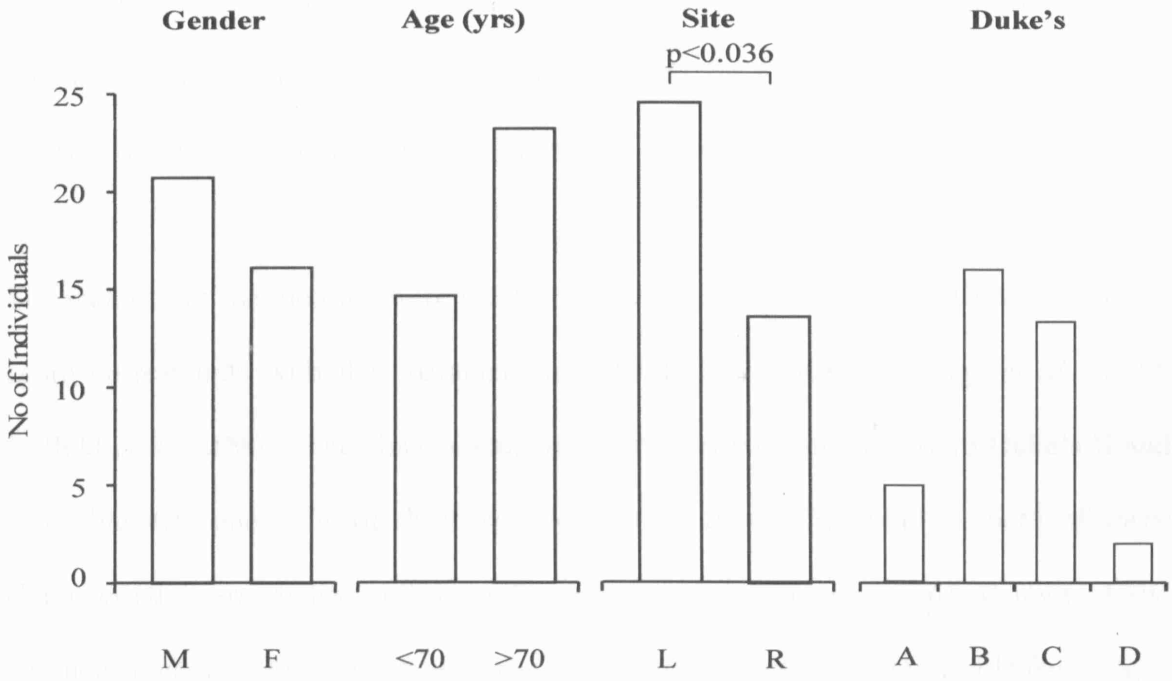


Gender		Age (years)		Site		Duke's Stage			
Male	Female	< 70	> 70	Left	Right	A	B	C	D
12 (67%)	6 (33%)	10 (56%)	8 (44%)	12 (67%)	6 (33%)	1 (6%)	9 (50%)	6 (33%)	2 (11%)

Figure 4.2
Distribution of gender, age, site of tumour and Duke's stage of MSI-L tumours. The numbers represent total numbers with percentages of group are shown in parenthesis.

MSS tumours

MSS tumours, 16 (43%) were females and 21 (57%) were males (Figure 4.3, $p>0.05$, Pearson Chi-square test). Fourteen (14; 38%) patients were less than 70 years old and 23 (62%) were older than 70 years (Figure 4.3, $p>0.05$, Pearson Chi-square test). Thirteen (13; 35%) of the MSS tumours were located in the right colon and 24 (65%) were in the left (Figure 4.3, $p<0.036$, Pearson Chi squared test). MSS and Duke's stage, 5 (14%) Duke's A, 16 (43%) B, 14 (38%) C, 2 (5%) D (Figure 4.3, $p>0.05$, Pearson Chi-squared test).



Gender		Age (years)		Site		Duke's Stage			
Male	Female	< 70	> 70	Left	Right	A	B	C	D
21	16	14	23	24	13	5	16	14	2
(57%)	(43%)	(38%)	(62%)	(65%)	(35%)	(14%)	(43%)	(38%)	(5%)

Figure 4.3
Distribution of gender, age, site of tumour and Duke's stage of MSS tumours. The numbers represent total numbers with percentages of group are shown in parenthesis.

4.5 Discussion

This sample of CRC has an almost equal distribution between genders (Table 4.1), which is in line with the characteristics of sporadic CRC (Silverberg, 1981). The median age of this sample of CRC is 72 years which is older than the common age for the disease, which is between 60 and 69 years (Goligher, 1941, 1984; Chi *et al.*, 1994). The older age of this sample group maybe due to the local population, where the samples were collected from. Analysis of the age of the sample population was undertaken by dividing the ages into two groups, above and below 70 years as Chao *et al.* (2000) found that MSI is more common in patients older than 70years. This age was also chosen as it was the median age at diagnosis for CRC from the Thames cancer registry which these samples were obtained.

The majority of the tumours were located in the left side of the colon (Table 4.1) which again corresponds with the characteristics of CRC as a whole (Maigs *et al.*, 1977; Loffeld *et al.*, 1996). The Duke's stage of the majority of tumours were Duke's B and C (Table 4.1) and 17% of these samples were MSI-H (Figure 4.1), both of these characteristics are again in line with the expected phenotypes of sporadic CRC (Barnetson *et al.*, 2000). There was also no correlation between MSI and Duke's stage. Overall, the samples have corresponding characteristics with CRC as a whole, which enables them to be representative of CRC and as such, are useful to investigate the properties of CRC with MSI.

The MSI-H tumours in this study were predominantly in female, older patients and the tumours were in the right colon (Figure 4.1). These results agree with published characteristics of MSI-H tumours (Chao *et al.*, 2000, Wright *et al.*, 2000, Gryfe *et al.*,

2001, Ward *et al.*, 2001). Conversely, MSI-L tumours were predominantly male but there was no significant difference in ages (Figure 4.2). In addition, the predominant location of MSI-L tumours was in the left colon (Figure 4.2 as reported by others (Jass *et al.*, 1999). MSS tumours had no gender difference but they were predominantly found in older patients and located in the left colon (Figure 4.3). This again agrees with the characteristics or sporadic CRC (Maigs *et al.*, 1977, Silverberg, 1981, Loffeld *et al.*, 1996).

The characteristics of the MSI-H within this sample, agrees with previous published results, which enables them to be used as representatives of MSI tumours. The differences in phenotype between MSS and MSI-L tumours means that the two groups of tumours were analysed separately, as it may reflect differences in the genotype of the tumours.

The normal samples have a similar gender distribution and the median age is the same median age which sporadic CRC develops. The majority of tumours which were resected were located in the left colon and where Duke's B and C. Although normal samples matched to tumour samples characteristics are the ideal samples to use as a comparison, this collection of normal samples can be used as an indicator of how tumour samples differ to normal bowel.

Summary

1. This sample of CRC is representative of CRCs as a whole and so are useful to investigate the properties of CRC with MSI.
2. There is no correlation between MSI and Duke's stage

CHAPTER 5

GENE TRANSCRIPTION

CHAPTER 5

5.1 Background

Central to the development, growth and spread of tumours are the processes of angiogenesis (Carmeliet & Jain, 2000) and lymphogenesis (Stacker *et al.*, 2000), both of which are mediated by members of the VEGF family of protein isoforms. $VEGF_A$ is a key protein involved in the initiation of angiogenesis, being stimulated in response to a developing hypoxia occurring as a tumour grows (Schweiki *et al.*, 1992, Adamis *et al.*, 1994, Aiello *et al.*, 1994). As such, altered levels of $VEGF_A$ mRNA may vary the level of tumour angiogenesis, and so influence the subsequent tumour development (Algire, 1945, Ide *et al.*, 1939). The soluble and functional isoforms of $VEGF_A$ are $VEGF_{121}$ and $VEGF_{165}$, and which are the isoforms thought to control the levels of angiogenesis (Houck *et al.*, 1992). The relationship between VEGF, angiogenesis and cancer is evident. Adenomas and CRC have an increased levels of VEGF protein compared to normal colonic mucosa (Wong *et al.*, 1999, Andre *et al.*, 2000), and VEGF protein expression would seem to correlate with tumour size, blood vessel involvement, histology and Duke's stage (Hanrahan *et al.*, 2003). However, although MSI-H tumours have been shown to have a decreased level of VEGF compared to MSS tumours (Wynter *et al.*, 1999), the relevance or relationship of angiogenesis and VEGF in the mechanism(s) underlying CRC with MSI are unclear. Furthermore, although gene mutation is clearly implicated in tumour development, it is also apparent that the activity of genes such as over- or under-expression can be similarly tumourigenic. The investigation of gene transcription can identify changes in gene activity. So, examining the levels of gene transcription of $VEGF_{121}$ and $VEGF_{165}$ may help to clarify the

relationship between VEGF and the processes of angiogenesis in CRC with MSI, and potentially therefore, a mechanism to explain the differing phenotypes of MSI tumours.

$VEGF_C$ is involved in the regulation of lymphangiogenesis which is implicated in tumour spread (Stacker *et al.*, 2002). Hence the level of gene transcription of $VEGF_C$ may also influence the spread of tumours (Akagi *et al.*, 2000). Therefore, as with $VEGF_A$ discussed above, examining the transcription (activity) of $VEGF_C$ may help to clarify the processes of lymphangiogenesis in CRC with MSI and a potential mechanism to explain MSI tumour phenotypic differences.

Gene transcription was analysed according to the demographics of the sample tumours, and also compared to controls as a means to examine any potential relationship with the characteristics of MSI tumours.

Typically, CRC are examined for MSI by a panel of five 'markers' consisting of both mononucleotide and dinucleotide repeat regions (National Cancer Institute, 1998). Jass *et al.*, 1998/99 suggested that true MSI-L tumours are those with only unstable dinucleotide markers rather than National Cancer Institute suggested '<30-40%' of either mononucleotide and/or dinucleotide unstable markers. Therefore, to further investigate the relationship between VEGF and MSI, the transcription of the $VEGF_{121}$, $VEGF_{165}$, and $VEGF_C$ genes was examined separately according to the number and type of markers (mono- or di-nucleotide) that were unstable within each sample.

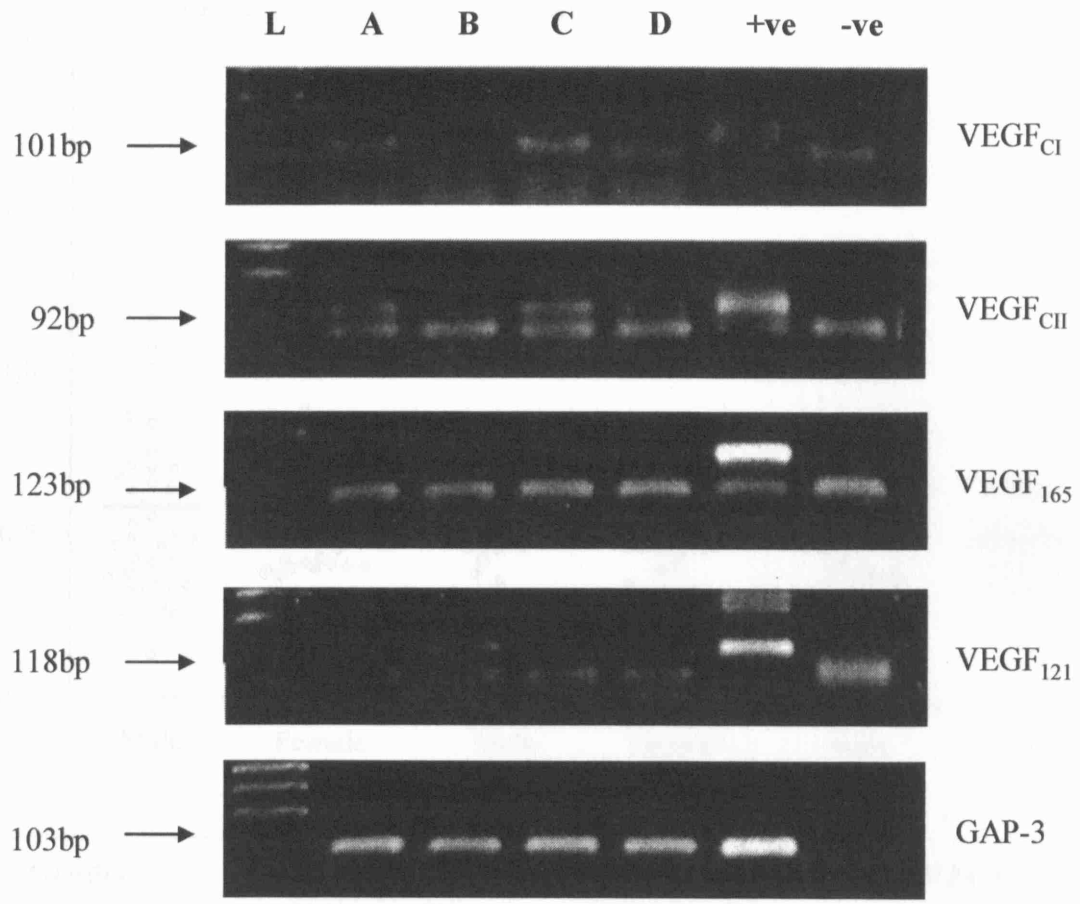
5.2 Aim

To determine the relationship between the transcription of $VEGF_{121}$, $VEGF_{165}$, and $VEGF_C$ and the gender, age of patient, tumour site, Duke's stage and MSI status of the study samples. Hence, to examine the relevance of VEGF gene transcription and so angiogenesis and lymphangiogenesis as a mechanism in the development of CRC with MSI.

5.3 Methods

Wax-embedded blocks of CRC were used as these were ready available samples which could be analysed. Total cellular RNA was extracted from wax-embedded CRC by an adaptation of the protocol for the paraffin block RNA isolation kit (Ambion Inc., Texas, USA, Section 3.3.1). The lengths of total cellular RNA extracted from wax blocks are smaller (100bp) than if extracted from fresh samples (200-300bp). The concentration of RNA extracted was calculated by measuring the light absorbance of nucleotides in solution at the ultraviolet region of the spectrum using a spectrophotometer (UV 1101 Biotech Photometer, WPA, UK). The RT technique (Section 3.3.3) was used to produce cDNA and this method involves denaturation of secondary structures which RNA can exhibit, addition of a random hexanucleotide to prime reverse transcription at several points along the transcript and reverse transcription to produce full length cDNA. The PCR technique (Section 3.3.1) was used to amplify the small fragments of cDNA sequence to produce multiple copies. In order to achieve this primers were designed to amplify the smaller gene mRNA products. In addition a 'step up' PCR cycling protocol (Table 3.6) was required to achieve optimal amplication of the genes. The amplified PCR products were then visualised by agarose gel electrophoresis. The gels were visualised under uv light and a Polaroid photograph was taken. The resulting

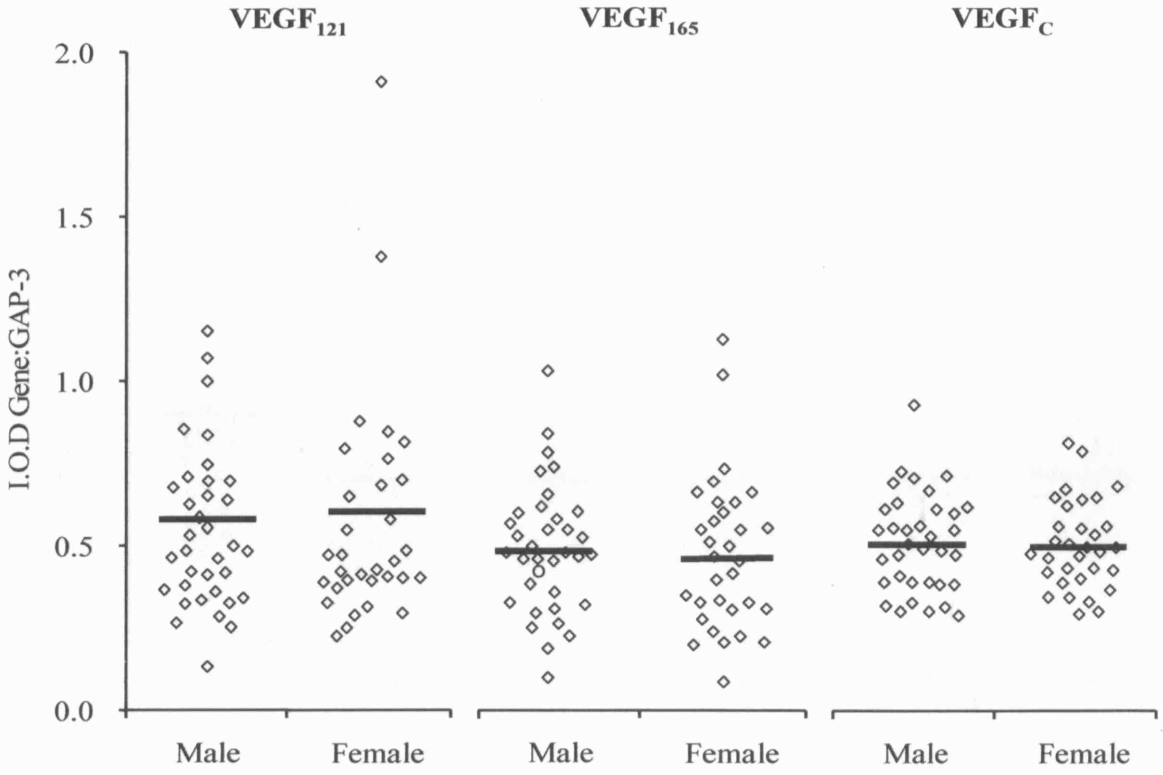
photograph of the gel electrophoresis was analysed to determine the IOD of each band and the level of gene transcription was estimated in a semi-quantitative manner as a ratio of the IOD of the gene compared to GAP-3, the internal control. Picture 5.1 shows a representative picture of the PCR products used for analysis of $VEGF_{121}$, $VEGF_{165}$, $VEGF_{CI}$, and $VEGF_{CII}$ level of RNA by scanning densitometry.



Picture 5.1
Representative picture of PCR products of $VEGF_{CI}$, $VEGF_{CII}$, $VEGF_{121}$, and $VEGF_{165}$. A to D are different patient samples. L represents 100bp ladder which gives an indication of product size. Numbers on the left side of the page are the product sizes of the respective PCR products. +ve and –ve are the controls. GAP-3 is the internal house keeping gene PCR products for the corresponding samples.

5.4 Results

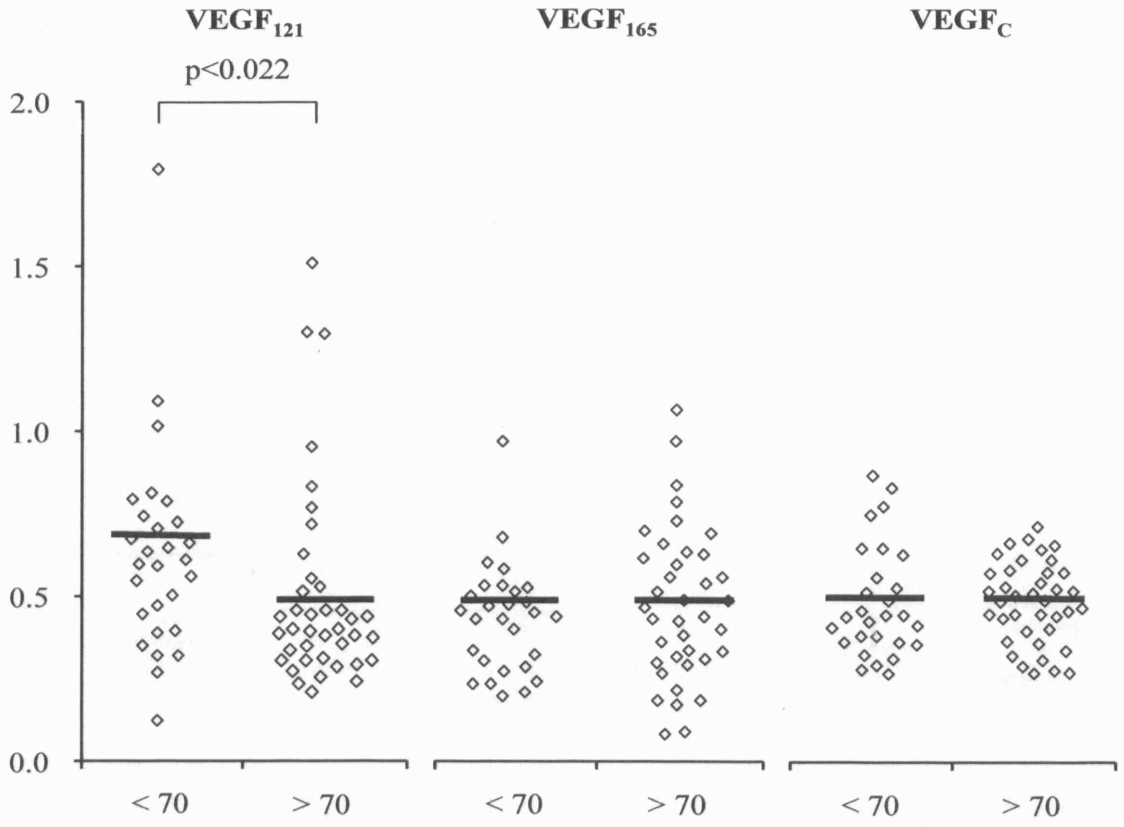
The raw data are presented in Appendices I and II. There was little or no difference between males and females in the levels of $VEGF_{121}$, $VEGF_{165}$, $VEGF_C$ mRNA (Nb, this should be transcription as the levels of mRNA are semi-quantitatively presented and have been normalised to GAP - they are not absolute measurements!) (Figure 5.1, $p>0.1$, ANOVA).



Gender	$VEGF_{121}$ mean (s.e.m)	$VEGF_{165}$ mean (s.e.m)	$VEGF_C$ mean (s.e.m)
Male	0.520 (0.037)	0.484 (0.029)	0.500 (0.022)
Female	0.539 (0.058)	0.462 (0.038)	0.492 (0.023)

Figure 5.1
Ratio of integrated optical density (IOD) of $VEGF_{121}$, $VEGF_{165}$, and $VEGF_C$ to the house keeping gene GAP-3 in CRC according to gender. The table shows numbers which are the mean values of all patients within each group and the standard errors of the mean in parenthesis. —— represents mean I.O.D. gene:GAP-3.

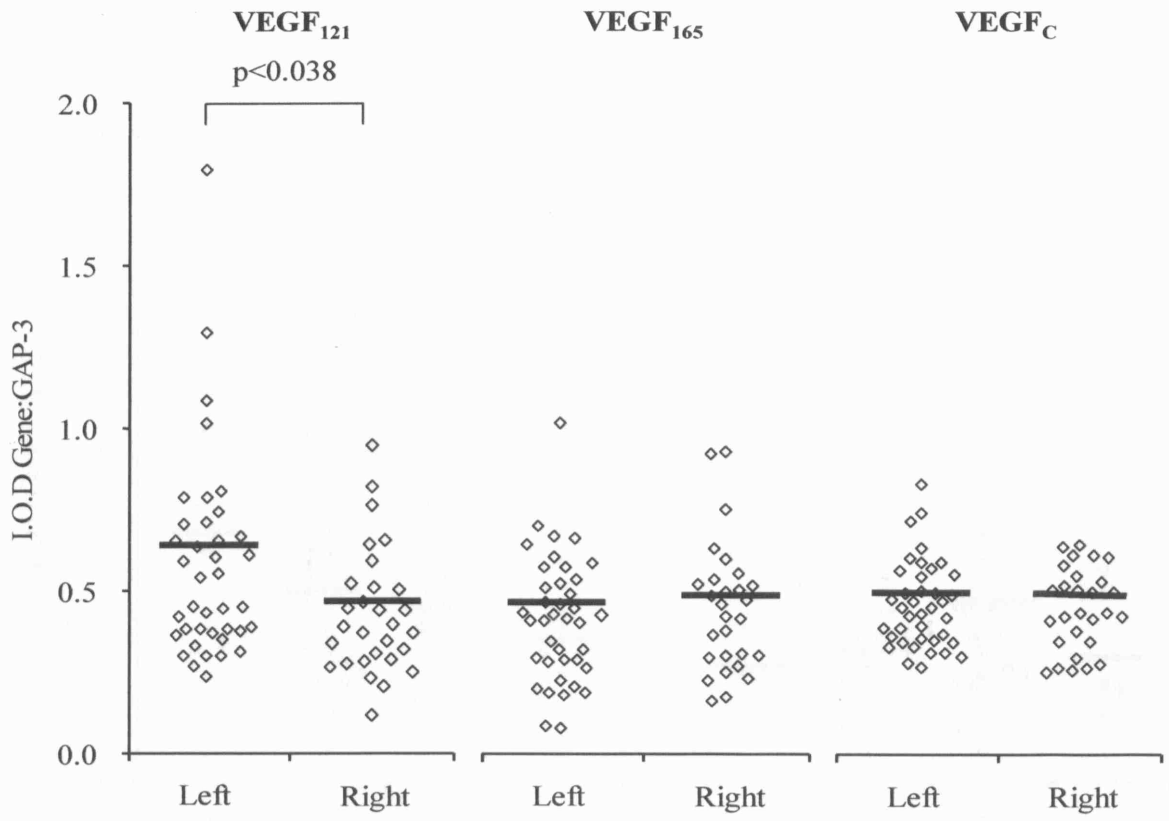
In contrast, for age, patients who were less then 70 years old, had an increased level of $VEGF_{121}$ transcription (Figure 5.2, $p<0.022$, ANOVA). There was little or no difference in the levels of $VEGF_{165}$ and $VEGF_C$ transcription with age (Figure 5.2, $p>0.1$, ANOVA).



Age (years)	$VEGF_{121}$ mean (s.e.m)	$VEGF_{165}$ mean (s.e.m)	$VEGF_C$ mean (s.e.m)
< 70	0.648 (0.065)	0.429 (0.033)	0.493 (0.031)
> 70	0.484 (0.037)	0.482 (0.036)	0.491 (0.019)

Figure 5.2
Ratio of integrated optical density (IOD) of $VEGF_{121}$, $VEGF_{165}$, and $VEGF_C$ to the house keeping gene GAP-3 in CRC according to age. The table shows numbers which are the mean values of all patients within each group and the standard errors of the mean in parenthesis. _____ represents mean I.O.D. gene:GAP-3.

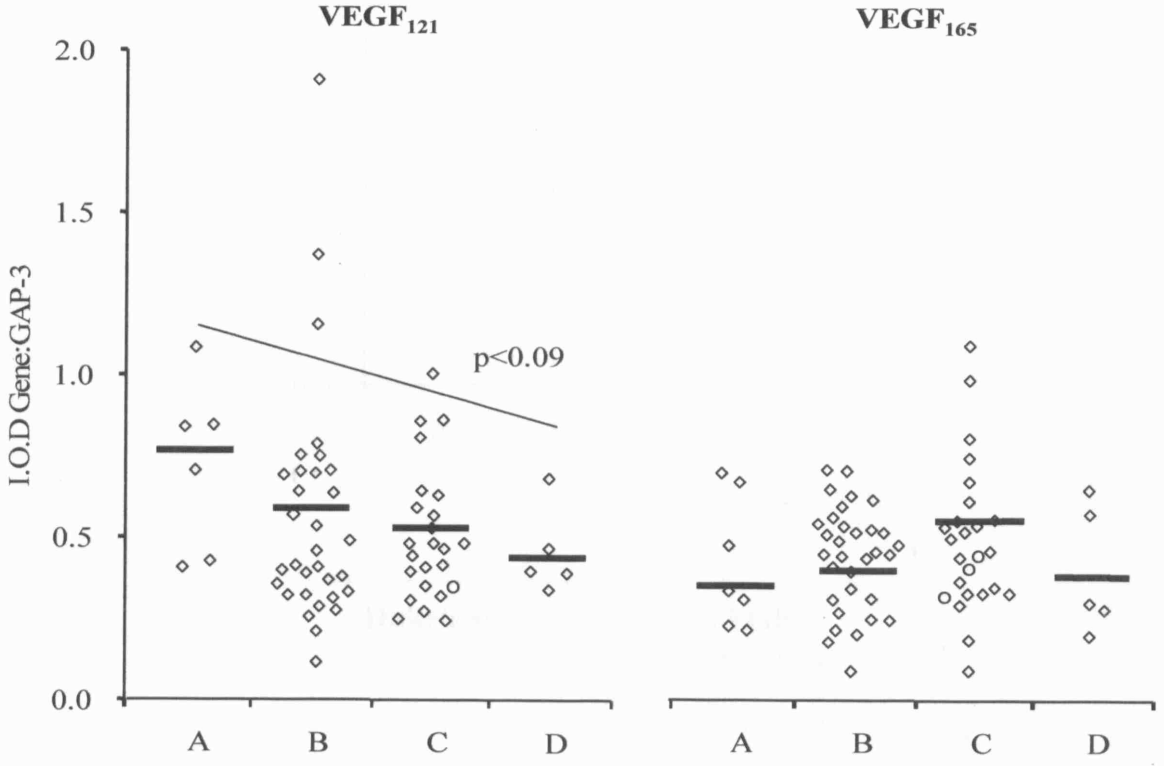
The level of $VEGF_{121}$ transcription was increased in left sided CRC than in right sided CRC (Figure 5.3, $p<0.038$, ANOVA) but the levels of transcription for $VEGF_{165}$ and $VEGF_C$ were not different (Figure 5.3, $p>0.1$, ANOVA).



Site	$VEGF_{121}$ mean (s.e.m)	$VEGF_{165}$ mean (s.e.m)	$VEGF_C$ mean (s.e.m)
Left	0.615 (0.053)	0.442 (0.033)	0.499 (0.023)
Right	0.465 (0.039)	0.485 (0.039)	0.482 (0.026)

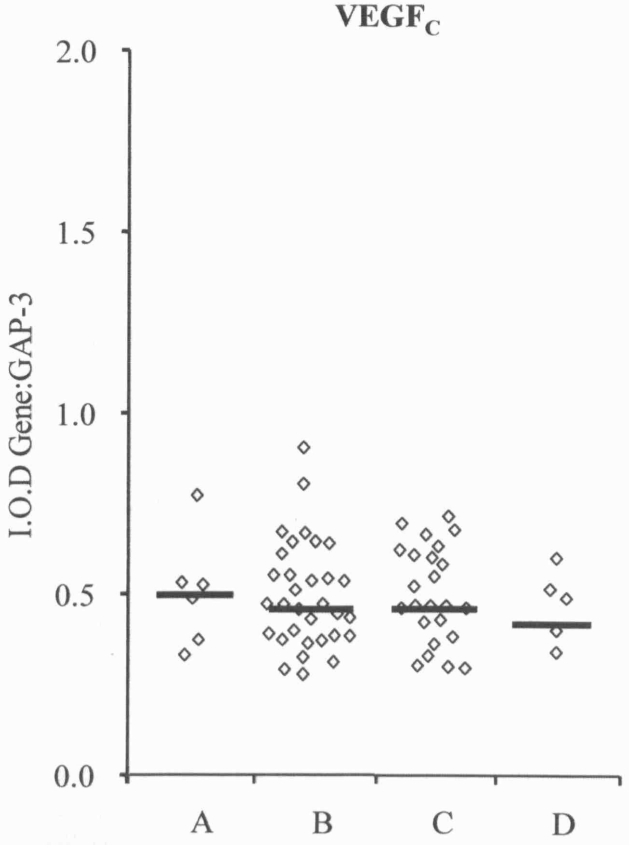
Figure 5.3
Ratio of integrated optical density (IOD) of $VEGF_{121}$, $VEGF_{165}$, and $VEGF_C$ to the house keeping gene GAP-3 in CRC according to site. The table shows numbers which are the mean values of all patients within each group and the standard errors of the mean in parenthesis. ____ represents mean I.O.D. gene:GAP-3.

There was an inverse correlation of $VEGF_{121}$ transcription with the highest level of transcription in Duke's A decreasing with Duke's stage and the lowest level was in Duke's D, but this did not reach statistical significance (Figure 5.4, $p<0.09$, ANOVA). There was no difference with $VEGF_{165}$ and $VEGF_C$ and Duke's stage (Figure 5.4 and 5.5, $p>0.1$, ANOVA).



Duke's stage	$VEGF_{121}$ mean (s.e.m)	$VEGF_{165}$ mean (s.e.m)
A	0.713 (0.108)	0.380 (0.073)
B	0.570 (0.063)	0.437 (0.029)
C	0.510 (0.042)	0.523 (0.052)
D	0.451 (0.060)	0.400 (0.089)

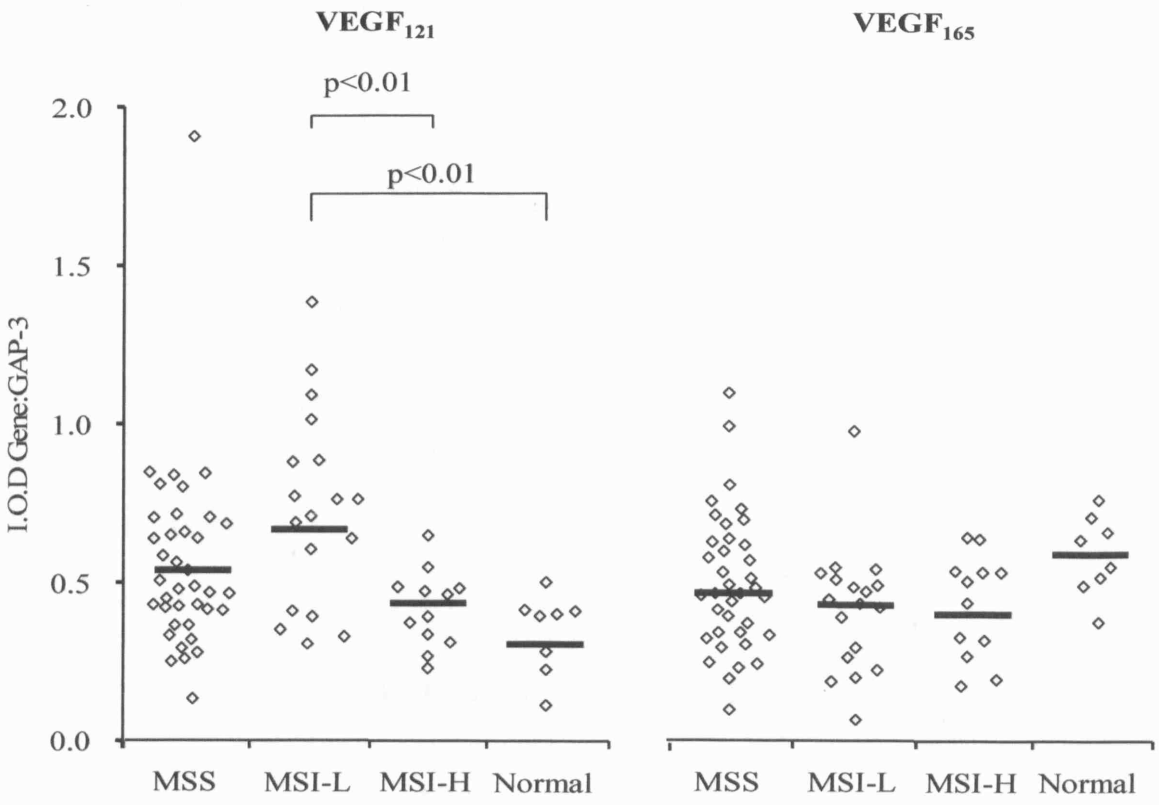
Figure 5.4
Ratio of integrated optical density (IOD) of $VEGF_{121}$, and $VEGF_{165}$, to the house keeping gene GAP-3 in CRC according to Duke's stage. The table shows numbers which are the mean values of all patients within each group and the standard errors of the mean in parenthesis. —— represents mean I.O.D. gene:GAP-3.



Duke's stage	VEGF$_C$ mean (s.e.m)
A	0.504 (0.064)
B	0.493 (0.026)
C	0.494 (0.028)
D	0.464 (0.045)

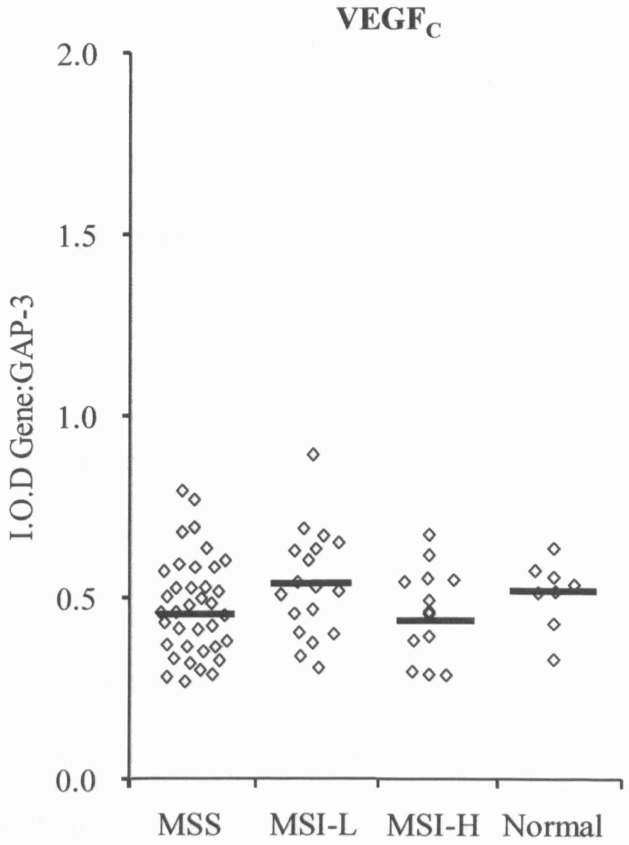
Figure 5.5
Ratio of integrated optical density (IOD) of VEGF$_C$ to the house keeping gene GAP-3 in CRC according to Duke's stage. The table shows numbers which are the mean values of all patients within each group and the standard errors of the mean in parenthesis. —— represents mean I.O.D. gene:GAP-3. $p>0.05$, ANOVA.

$VEGF_{121}$ transcription was significantly increased in MSI-L cancers compared to MSI-H (Figure 5.6, $p<0.010$, ANOVA). $VEGF_C$ transcription showed a similar trend, but this was not statistically significant (Figure 5.7, $p>0.05$, ANOVA). There was no significant difference in the levels of $VEGF_{165}$ according to MSI status (Figure 5.6, $p>0.1$, ANOVA).



MSI Status	$VEGF_{121}$ mean (s.e.m)	$VEGF_{165}$ mean (s.e.m)
MSS	0.538 (0.048)	0.491 (0.036)
MSI-L	0.679 (0.078)	0.428 (0.047)
MSI-H	0.409 (0.035)	0.413 (0.048)
Normal	0.331 (0.044)	0.592 (0.045)

Figure 5.6
Ratio of integrated optical density (IOD) of $VEGF_{121}$, and $VEGF_{165}$, to the house keeping gene GAP-3 in CRC according to microsatellite status. The table shows numbers which are the mean values of all patients within each group and the standard errors of the mean in parenthesis. —— represents mean I.O.D. gene:GAP-3.



MSI Status	VEGF$_C$ mean (s.e.m)
MSS	0.474 (0.022)
MSI-L	0.542 (0.036)
MSI-H	0.471 (0.035)
Normal	0.516 (0.034)

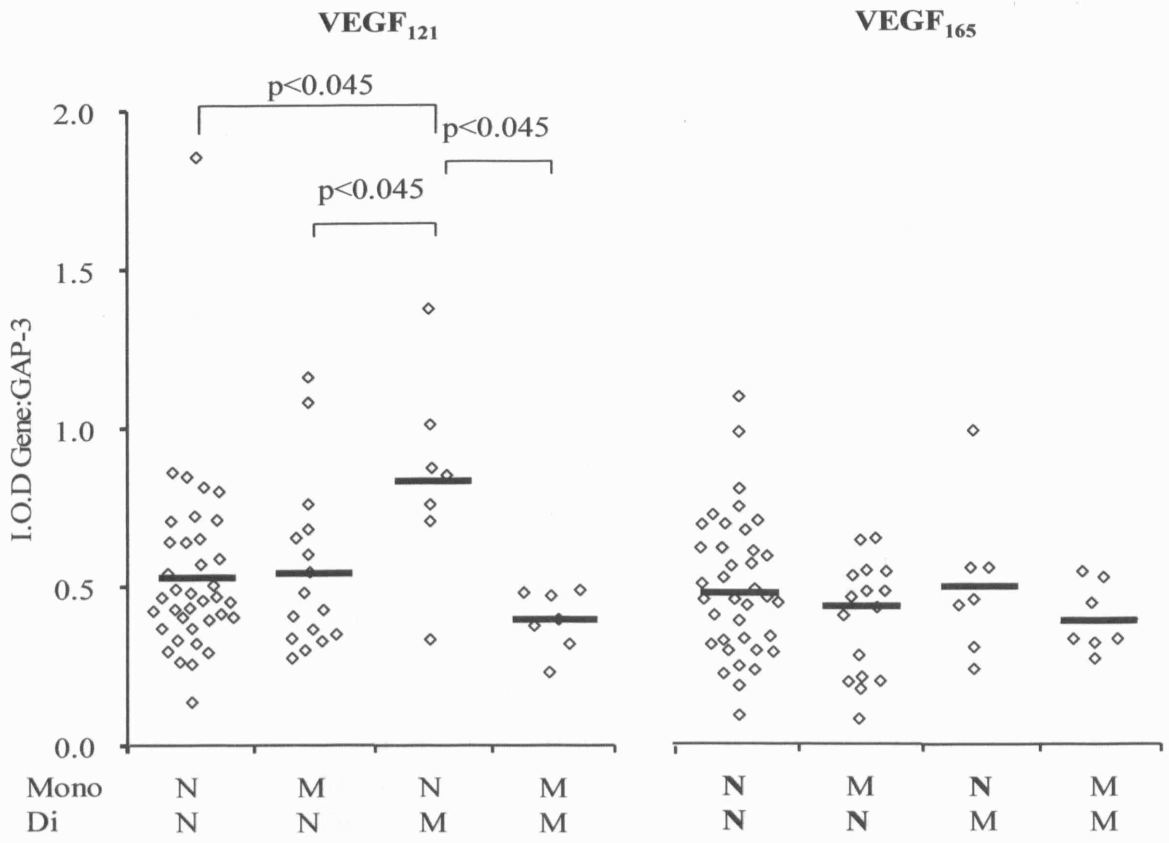
Figure 5.7
Ratio of integrated optical density (IOD) of VEGF$_C$ to the house keeping gene GAP-3 in CRC according to microsatelite status. The table shows numbers which are the mean values of all patients within each group and the standard errors of the mean in parenthesis. —— represents mean I.O.D. gene:GAP-3. $p > 0.05$, ANOVA.

There was a decreased level of $VEGF_{121}$ transcription in normal compared to MSI-L tumours ($p<0.01$, ANOVA) and MSS ($p<0.06$, ANOVA). MSI-H and MSS tumours had an increased level of $VEGF_{121}$ transcription when compared to normal, but this was not statistically significant. For $VEGF_{165}$, the highest level of transcription was found in normal colonic mucosa however, the difference in transcription was not significant when compared to tumours regardless of MSI status. The level of $VEGF_C$ transcription was not significantly different when comparing normal samples to MSS, MSI-L and MSI-H tumours (Figure 5.6 and 5.7).

It has been discussed that tumours with only mutations of dinucleotide markers are true MSI-L tumours. It was found that tumours with just mutated dinucleotide markers had increased levels of $VEGF_{121}$ and $VEGF_C$ transcription (Figure 5.8 and 5.9, $p<0.045$, ANOVA). There was little or no difference in levels of $VEGF_{165}$ transcription with different mutated markers (Figure 5.8, $p>0.1$, ANOVA).

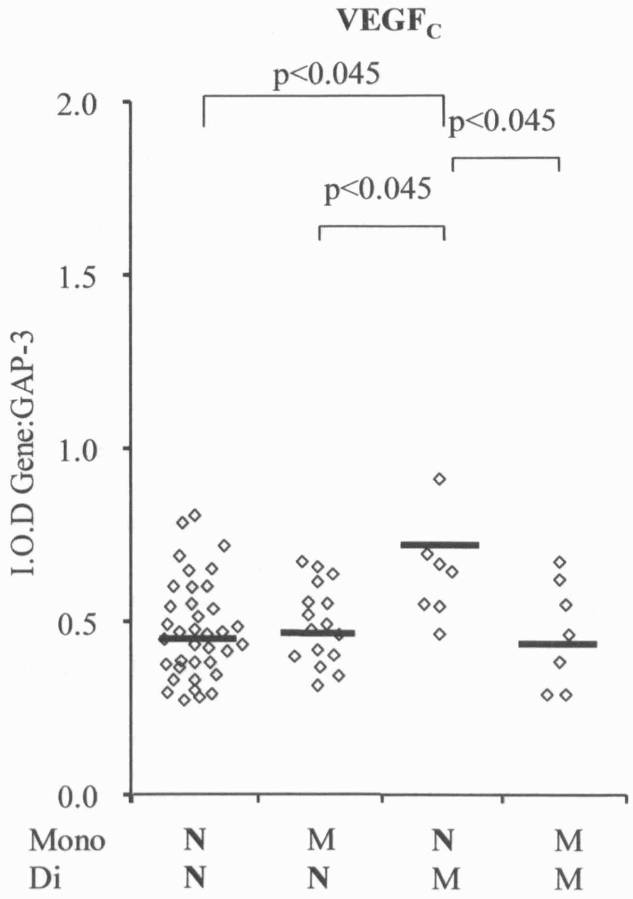
The levels of the front end of $VEGF_C$ gene compared to MSI were higher than the back end of the gene ($VEGF_{CI}$ – MSS 0.317, MSI-L 0.385, MSI-H 0.337; $VEGF_{CII}$ MSS 0.653, MSI-L 0.735, MSI-H 0.637, $p < 0.05$, Students unpaired T test). However, the trend in levels of the $VEGF_C$ comparing front and back of the gene were the same and so the results of both ends of the genes were combined to measure the whole gene.

Investigating the levels of RNA as a whole, in the tumour samples, it was found that $VEGF_{165}$ had a positive correlation with $VEGF_C$ ($p<0.05$, Pearson's Correlation). In addition $VEGF_{121}$ had a negative correlation to age and site of tumour ($p<0.05$, Pearson's Correlation).



Nucleotide markers	**$VEGF_{121}$** mean (s.e.m)	**$VEGF_{165}$** mean (s.e.m)
NN	0.538 (0.048)	0.491 (0.036)
MN	0.538 (0.067)	0.400 (0.041)
NM	0.841 (0.120)	0.506 (0.092)
MM	0.375 (0.040)	0.387 (0.061)

Figure 5.8
Ratio of integrated optical density (IOD) of $VEGF_{121}$, and $VEGF_{165}$, to the house keeping gene GAP-3 in CRC according to mutations of mononucleotide and dinucleotide markers. The table shows numbers which are the mean values of all patients within each group and the standard errors of the mean in parenthesis. —— represents mean I.O.D. gene:GAP-3. M represents mutation of nucleotide marker. N represents no mutations of nucleotide markers.



Nucleotide markers	**VEGF$_C$** mean (s.e.m)
NN	0.504 (0.064)
MN	0.493 (0.026)
NM	0.494 (0.028)
MM	0.464 (0.045)

Figure 5.9
Ratio of integrated optical density (IOD) of VEGF$_C$ to the house keeping gene GAP-3 in CRC according to mutations of mononucleotide and dinucleotide markers. The table shows numbers which are the mean values of all patients within each group and the standard errors of the mean in parenthesis. ____ represents mean I.O.D. gene:GAP-3. M represents mutation of nucleotide marker. N represents no mutations of nucleotide markers.

5.5 Discussion

Using the technique of extracting RNA from wax-embedded tumour samples, as a substrate for RT-PCR, enables research to occur without relying on fresh tumour samples. It also enables the use of a potentially large numbers of archival samples which are more freely available.

The discovery of MSI has prompted investigating alternative pathways in the pathogenesis of CRC (Markowitz *et al.*, 1995, Fujiwara *et al.*, 1998, Jass *et al.*, 1998, Jass *et al.*, 1999, Barnetson *et al.*, 2000, Chao *et al.*, 2000, Bacon *et al.*, 2001, Goel *et al.*, 2001, Young *et al.*, 2001, Jass, 2002, Jass *et al.*, 2002). Studies examining mutations of genes and the expression of protein, provides an earlier insight into the pathogenesis of CRC.

$VEGF_C$ is secreted in an inactive form and becomes active by proteolytic cleavage of the protein (Joukov *et al.*, 1996, Achen *et al.*, 1998, Joukov *et al.*, 1997, Stacker *et al.*, 1999). Investigation of gene transcription again looks further upstream in the pathway resulting in the development of a tumour. It also allows a comparison of the level of RNA and the resulting protein expression of $VEGF_C$. If there is no correlation between gene transcription and protein expression, it may indicate that post transcriptional or translational events are responsible for differences in protein expression of $VEGF_C$.

There was no difference in levels of transcription of $VEGF_{121}$, $VEGF_{165}$ and $VEGF_C$ according to gender of the patient. This indicates that altered levels of angiogenesis and lymphogenesis does not explain the increased incidence of CRC in males.

MSI-H tumours are predominantly found in older people (Chao *et al.*, 2000, Young *et al.*, 2001), corresponding with this, lower levels of $VEGF_{121}$ transcription were found in patients older than 70 years old. In contrast, $VEGF_{165}$ transcription was increased in patients older than 70 years old. Increasing age may decrease the potential to up regulate $VEGF_{121}$ to stimulate angiogenesis and so CRC have less potential to grow and so have a better prognosis.

MSI-H tumours are also found predominantly found in right colon cancer (Chao *et al.*, 2000, Gryfe *et al.*, 2001). There is evidence that CRC, which develop on the left side of the colon, have a different aetiology to tumours which arise on the right side of the colon. This study agrees with this theory, which found that $VEGF_{121}$ transcription was decreased in right colon cancer. This again corresponds with MSI-H tumours developing predominantly in right colon cancer. This may indicate that the altered aetiology could involve an alteration in the level of angiogenesis of the CRC. In support of this COX_2, which is also involved in the angiogenesis of CRC, has been found to be decreased in right CRC (Sheng *et al.*, 1997, Tsujii *et al.*, 1997, Tsujii *et al.*, 1998, Fujisaki *et al.*, 1998).

$VEGF_{121}$, interestingly, was found to have a trend towards an inverse correlation with Duke's stage. Duke's A has the highest level, which may reflect that upregulation of $VEGF_{121}$ occurs early in the development of a CRC. This agrees with a previous study of adenomas and CRC, which found there were increased levels of VEGF in both tubular adenomas and CRC (Hanrahan *et al.*, 2003). It also agrees with evidence that $VEGF_A$ is required to instigate angiogenesis, which occurs early in the development of a tumour. For a tumour to grow larger than a simple collection of tumour cells,

angiogenesis is required and $VEGF_{121}$ may be the VEGF isoform which is responsible for instigating angiogenesis in CRC. Duke's D has the lowest level of $VEGF_{121}$ which could reflect that the instigation of angiogenesis is no longer required, as the tumour has grown and spread, and so there is no corresponding upregulation of transcription of $VEGF_{121}$.

Analysing gene transcription according to MSI status, there was a lower level of $VEGF_{121}$ transcription in MSI-H tumours compared to MSI-L tumours. This could be due to decreased levels of transcription of $VEGF_{121}$ in MSI-H tumours. However, when comparing with normal colon samples the level of $VEGF_{121}$ transcription is not significantly different from MSI-H tumours. This could then be explained by a lack of stimulus, in MSI-H tumours, to cause an upregulation of transcription of $VEGF_{121}$, which occurs in MSS and MSI-L tumours. The decreased level of $VEGF_{121}$ may indicate that there is a lower level of angiogenesis occurring in MSI-H tumours and so explain in part the improved prognosis and reduce spread of MSI-H tumours. COX_2, also involved in angiogenesis of CRC, has been found to have similar differences in levels of COX_2 when comparing the levels of gene with MSI-L and MSI-H tumours (Karnes *et al.*, 1998).

$VEGF_{165}$, there was no significant difference in the level of transcription with MSI status. Comparing the tumours to the normal samples there was a significant difference in levels of transcription of MSI-L and MSI-H compared to normal colon samples. There was a decrease in the level of $VEGF_{165}$ in MSI-L and MSI-H tumours compared to normal tissue. This is in contrast to $VEGF_{121}$ where transcription increases in

tumours compared to normal colon. The process of tumour genesis may involve swiching off $VEGF_{165}$ and upregulating $VEGF_{121}$.

The decrease in level of $VEGF_{165}$ in MSI-L tumours maybe related to the aetiology of MSI-L tumours. The decrease in $VEGF_{165}$ soluble isoform, which is in contrast to the increase in $VEGF_{121}$, maybe explained due to preferential upregulation of an alternative splice variant. The immediate environment of the tumour is hypoxic which may preferentially stimulate the acidic isoform $VEGF_{121}$. This isoform may be the important splice variant which brings about a functional change in the level of angiogenesis and produce new blood vessels to enable the tumour to grow.

For $VEGF_C$, there was a trend towards increased level of transcription in MSI-L tumours. The lack of increase in MSI-H tumours may reflect a decreased level of lymphangiogenesis occurring and so explain why they have decreased lymphatic spread and improved prognosis. There was no significant difference in the levels of $VEGF_C$ in the tumour samples compared to the normal samples. $VEGF_C$ is secreted in an inactive protein and undergoes proteolytic cleavage to become active. This pathway maybe the important step in determining the amount of active $VEGF_C$ which is available to regulate the level of lymphangiogenesis rather than the level of gene transcription. The process could also be affected by post transcriptional events.

Jass *et al.* has provided much evidence that true MSI-L tumours are those with only mutated dinucleotide markers (Jass *et al.*, 2002). In order to investigate this further the results were reanalysed according to which markers were mutated. It was found that tumours with only mutated dinucleotide markers had a significant increase in the levels

of $VEGF_{121}$ and $VEGF_C$ transcription. This shows there are significant differences in gene transcription of factors involved in angiogenesis and lymphangiogenesis of MSI-L tumours. This indicates that MSI-L tumours are a distinct group from both MSS and MSI-H and is possibly due to an alternative development of CRC.

Analysing the levels of transcription as a whole, in the tumour samples, it was found that $VEGF_{165}$ had a positive Pearson's correlation with $VEGF_C$. This may mean that $VEGF_{165}$'s role is to facilitate the functions of $VEGF_C$ rather than having a functional role, for example, in instigating angiogenesis. This would mean that the actual level of $VEGF_{165}$ transcription is not important but that the gene is present. This also allows preferential upregulation of the functional soluble isoforms $VEGF_{121}$ and $VEGF_C$.

In conclusion, MSI-H tumours may have a better prognosis due to reduced angiogenic and lymphogenic potential. $VEGF_{121}$ isoform maybe responsible for the early development of a CRC and its initial spread. In contrast, $VEGF_{165}$ may be involved with subsequent spread or act as a facilitator for other ligands. CRC with only one mutated dinucleotide marker appear to be a distinct MSI-L group, at a molecular level, with characteristic levels of gene activity. A study of gene transcription allows closer interpretation of the differences of CRC with MSI, which aids explanation of the differences in their phenotypes.

Summary

1. Wax embedded tumour samples are a valuable source of mRNA which can be used as a substrate for RT-PCR.
2. MSI-H tumours were predominantly right colon cancers and were found in female

and older patients.

3. Lower levels of $VEGF_{121}$ transcription were found in older patients and right colon cancers.
4. Duke's A CRC had the highest level of $VEGF_{121}$ transcription when compared to Duke's D tumours.
5. MSI-H tumours had the lowest level of $VEGF_{121}$ transcription.
6. True MSI-L tumours were a distinct group from both MSS and MSI-H tumours.

CHAPTER 6

PROTEIN EXPRESSION

CHAPTER 6

6.1 Background

Lymphangiogenesis along with angiogenesis is central to the development, growth and spread of tumours and $VEGF_C$ is involved in the regulation of lymphangiogenesis (Stacker, 2002). The levels of $VEGF_C$ transcription has been examined to attempt to clarify the processes of lymphangiogenesis in CRC with MSI and a potential mechanism to explain the differences in MSI tumour phenotype. To further investigate this mechanism the levels of $VEGF_C$ protein expression were measured. $VEGF_C$ protein is secreted in an inactive form and becomes active by proteolytic cleavage of the propeptide. This means that a certain level of gene transcription may produce different levels of protein expression in different conditions (Joukov *et al.*, 1996, Joukov *et al.*, 1997, Achen *et al.*, 1998, Stacker *et al.*, 1999). CRC tumours of different MSI and Duke's stage may be explained in part by altered levels of $VEGF_C$ protein.

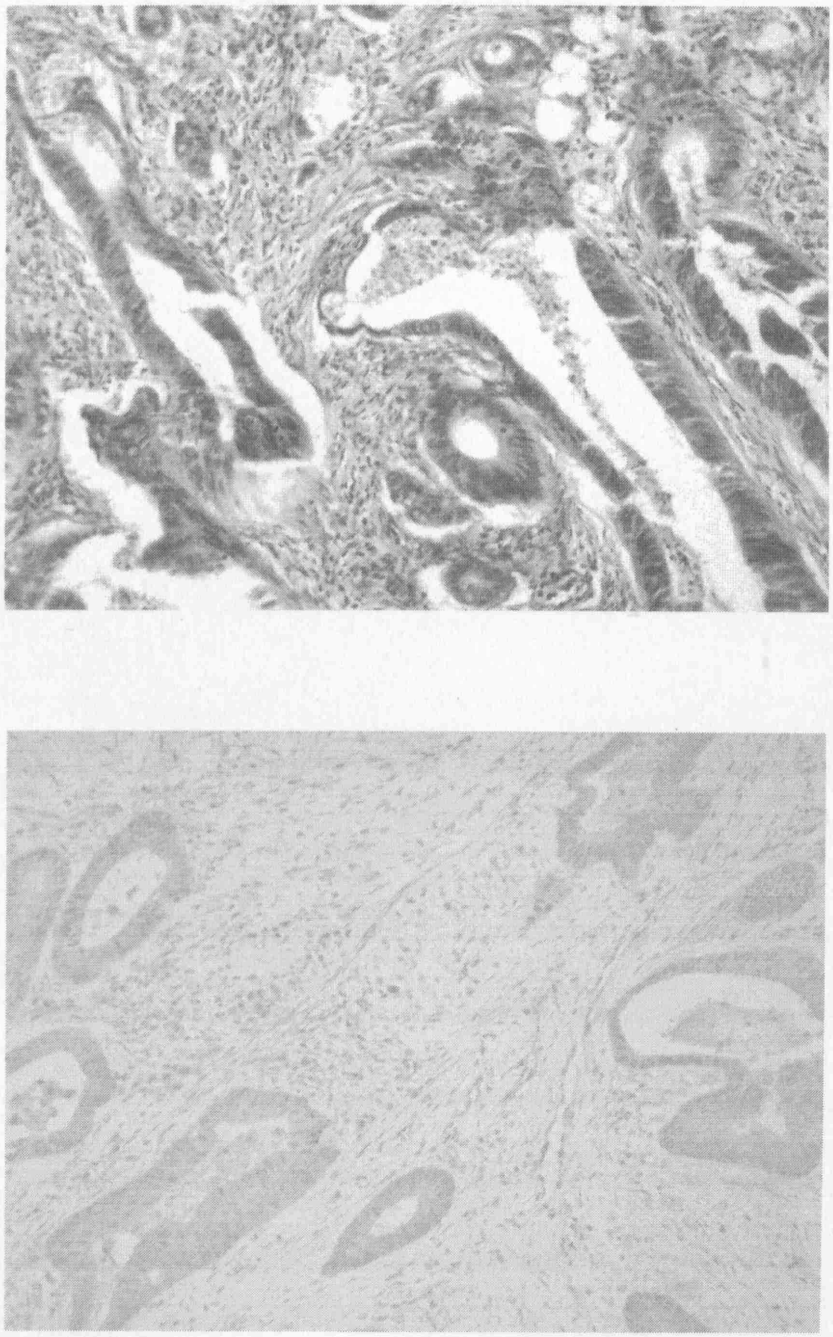
6.2 Aim

To determine the relationship between protein expression of $VEGF_C$ and the tumour site, Duke's stage and MSI status of the study samples.

6.3 Methods

Immunohistochemistry (Section 3.4.2), of wax embedded tumour blocks, was used to measure the levels of protein expression of $VEGF_C$. A polyclonal $VEGF_C$ antibody was used to detect the antigen. Antigen retrieval, using citrate buffer, and hydrogen peroxidase treatment was used. Non-specific binding was reduced by treating with normal serum, which blocks non-specific antigen binding sites. Avidin biotin staining

technique was used and the sections were counterstained with Mayers' haematoxylin (Tables 3.9, 3.10, 3.11). Positive staining was classified as staining in the cytoplasm. The positive result was analysed according to the percentage of cytoplasm which was stained. Negative was classified as no or unclear staining of cytoplasm (Picture 6.1).



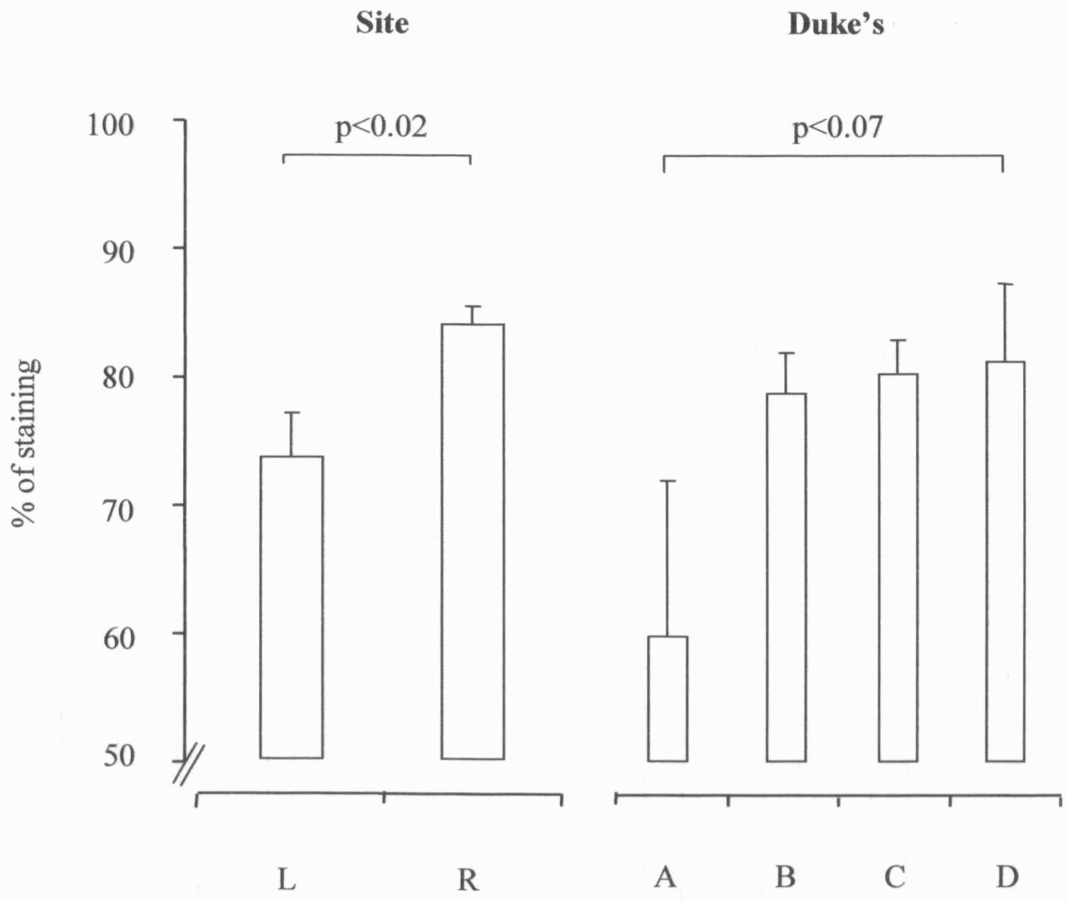
Picture 6.1
A is a picture of H & E staining of sample 40. **B** is a picture of staining of $VEGF_C$ protein expression of sample 40. Magnification x200.

6.4 Results

The majority of tumours (97%) stained positively for $VEGF_C$ protein. Therefore, in order to see if there was a difference in degree of staining, i.e. degree of protein expression, the percentage of staining of cytoplasm was measured and the results were then analysed according site of tumour, Duke's stage, MSI status and mutation of nucleotide marker.

The protein expression of $VEGF_C$ was analysed according to site of tumour. There was a higher expression of $VEGF_C$ in right sided tumours compared to left sided tumours (Figure 6.1, $p<0.02$, ANOVA).

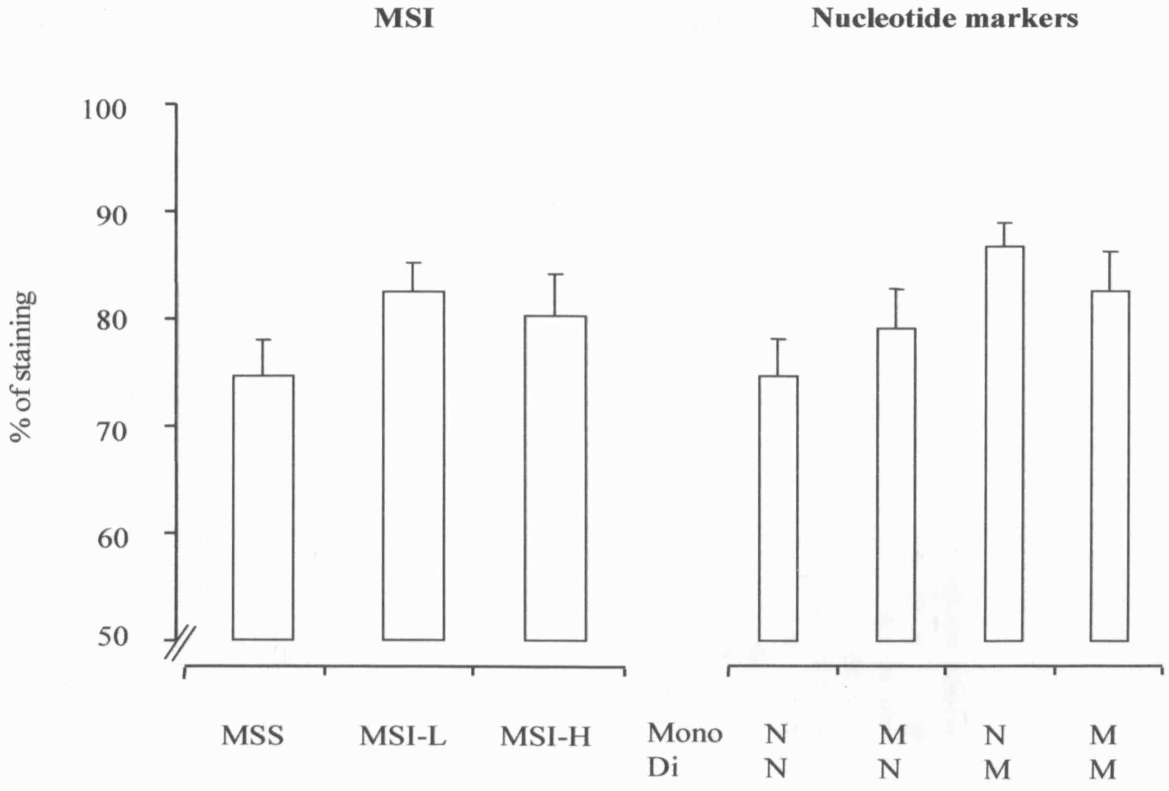
Investigating protein expression of $VEGF_C$ according to Duke's stage, the lowest protein expression was in Duke's A tumours and the highest in Duke's D (Figure 6.1, $p<0.075$, ANOVA).



	Site		Duke's Stage			
	Left	Right	A	B	C	D
% of staining	73.2	83.4	59.6	78.4	80.0	81.0
s.e.m	3.38	1.63	12.1	3.15	2.54	6.00

Figure 6.1
Percentage of staining for $VEGF_C$ protein according to the site of tumour and Duke's stage. The numbers represent percentage of staining of cytoplasm for $VEGF_C$ protein. Also shown is the standard error of the mean.

MSI-L tumours had the highest percentage of $VEGF_C$ protein expression, with MSS tumours having the lowest (Figure 6.2, $p>0.1$, ANOVA). This was also found when analysing the results according to which nucleotide markers were mutated. Tumours with only dinucleotide markers mutated, true MSI-L tumours, has the highest percentage of $VEGF_C$ protein expression (Figure 6.2, $p<0.07$, ANOVA).



	MSI			Nucleotide markers			
	MSS	MSI-L	MSI-H	NN	MN	NM	MM
% of staining	74.3	82.2	80.0	74.3	78.8	86.4	82.1
s.e.m	3.42	2.66	3.99	3.42	3.67	2.10	3.76

Figure 6.2
Percentage of staining for $VEGF_C$ protein according to MSI and which nucleotide markers were mutated. Mono = mononucleotide markers, Di = dinucleotide markers. N – nucleotide marker not mutated, M nucleotide marker mutated. The numbers represent percentage of staining of cytoplasm for $VEGF_C$ protein. Also shown is the standard error of the mean. $p>0.1$, ANOVA.

There was no correlation between the level of $VEGF_C$ gene expression and the percentage of $VEGF_C$ protein expression (Figure 6.3, $p>0.1$, Pearson's Bivariate correlation).

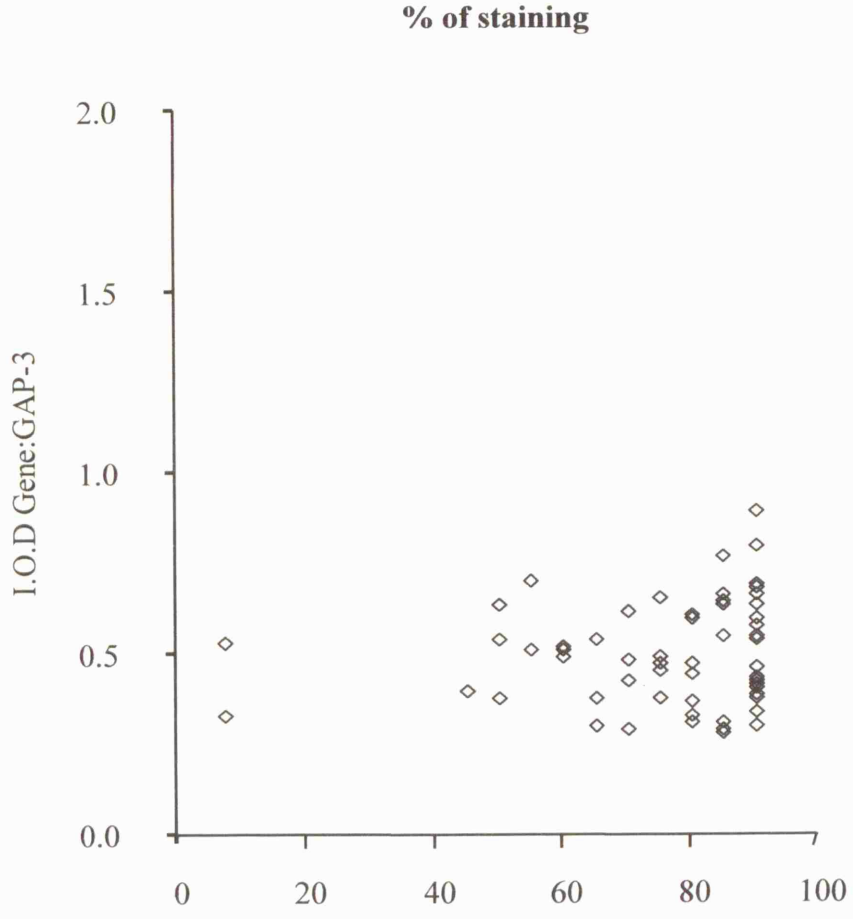


Figure 6.3
Ratio of integrated optical density (IOD) of $VEGF_C$ to the house keeping gene GAP-3 in CRC according to percentage of staining of cytoplasm with $VEGF_C$ protein. $p>0.1$, Pearson's Bivariate correlation.

6.5 Discussion

A higher protein expression of $VEGF_C$ in right sided tumours seems to contradict the finding that MSI-H tumours are more in right sided tumours and have a better prognosis (Wright *et al.*,2000). It would have been expected that there was a lower expression $VEGF_C$ in right sided tumours which may indicate that less lymphogenesis is occurring. This result is also opposite to the finding that there was decreased $VEGF_{121}$ gene expression in right sided tumours, which may reflect a decrease in angiogenesis. It maybe that the expressed $VEGF_C$, which is detected by immunohistochemistry, does not reflect the functional active $VEGF_C$, which is brought about by proteolytic cleavage of the secreted protein (Joukov *et al.*, 1996; Achen *et al.*, 1998; Joukov *et al.*, 1997; Stacker *et al.*, 1999).

The lowest protein expression of $VEGF_C$ is in Duke's A tumours especially when compared to Duke's C and D tumours. The relationship was not significant but this maybe due to small numbers of Duke's A tumours ($p<0.07$, ANOVA). This result is also consistent with work by Furudoi *et al.* (2002), which showed an increase in $VEGF_C$ at the deepest level of Duke's D tumours. It could be suggested that there is more expression of $VEGF_C$ protein in Duke's C and D tumours as lymph node spread and distant metastasis has occurred (Turnbull *et al.*, 1967). It has been previously demonstrated that $VEGF_C$ is positively correlated with lymph node involvement (Akagi *et al.*,2000; Neuchrist *et al.*, 2002; Hansel *et al.*, 2003; Nakamura *et al.*, 2003; Tanaka *et al.*, 2003).

Comparing $VEGF_C$ protein expression to MSI and mutations of nucleotide markers there was no definite correlation between the two. However, the highest level of

$VEGF_C$ protein expression was in MSI-L tumours and especially the true MSI-L tumours with only mutated dinucleotide markers.

The lack of correlation between protein expression and gene transcription of $VEGF_C$ could be due to several reasons. Firstly, the difference maybe due to post transcriptional or post translational changes. Post translational changes are known to occur as proteolytic cleavage is known to occur in order to make the $VEGF_C$ protein active. In support of this, it has been shown that induction of $VEGF_C$ mRNA is independent of protein synthesis and so the lack of correlation is to be expected (Engholm *et al.*, 1997; Ristimaki *et al.*, 1998; Tang *et al.*, 2003).

It is not known whether the immunohistochemical staining of protein $VEGF_C$ stains for the active or inactive form or a combination of the two. It may have been more informative to have measured the end product of the action of $VEGF_C$, i.e. the number of lymph vessels, rather than the protein itself. This could have been done by measuring lymphatic vessel density using the lymphatic endothelial marker LYVE-1 (Dadras *et al.*, 2003). Alternatively, measuring Flt-4, the receptor for $VEGF_C$ may give a better perception of active $VEGF_C$ protein. The level of Flt-4 may however, be affected by inducing factors other than $VEGF_C$ and so not give an accurate reflection of $VEGF_C$.

Alternatively, the lack of significant results maybe due to the technique of using wax embedded samples. It may have been more informative to analyse fresh samples for $VEGF_C$ immuno-reactivity.

In conclusion, there was no significant evidence that $VEGF_C$ protein is an indicator of severity or prognosis of a CRC or the level of lymphangiogenesis. The lack of correlation between gene expression and protein of $VEGF_C$ may reflect that measurement of protein is not the optimum method to measure lymphangiogenesis.

Summary

1. The highest $VEGF_C$ protein expression was in right colon cancers.
2. The highest $VEGF_C$ protein expression was in Duke's D compared to Duke's A.
3. There was no correlation between protein expression and gene transcription of $VEGF_C$.

CHAPTER 7

BLOOD VESSEL DENSITY

CHAPTER 7

7.1 Background

Angiogenesis is important for tumour development, growth and spread and it has already been discussed that $VEGF_A$ stimulates angiogenesis. $VEGF_{121}$ and $VEGF_{165}$ are soluble functional isoforms and this makes it difficult to get a true indication of their levels of protein production within wax embedded tissue as measurement of the protein expression of $VEGF_A$ is more likely to detect the insoluble storage isoforms $VEGF_{189}$ and $VEGF_{206}$. Therefore using immuno-histochemistry to detect $VEGF_A$ protein levels in the fixed samples does not provide an appropriate analysis, or a correct comparison with the levels of gene transcription of the soluble isoforms. An alternative is to measure the end product of the soluble VEGF isoforms by measuring the number of blood vessels formed by angiogenesis which are stimulated by the soluble isoforms. This technique uses immuno-histochemistry with an antibody against CD34 to detect blood vessels and so enable measurement of the blood vessel density. This indicates the level of angiogenesis which has been stimulated by the soluble isoforms of $VEGF_A$ (Kim *et al.*, 1993; Borgstrom *et al.*, 1996). It is therefore assumed that the higher the blood vessel density indicates a greater level of angiogenesis which has been stimulated by an increased protein level of the soluble $VEGF_A$ isoforms.

7.2 Aim

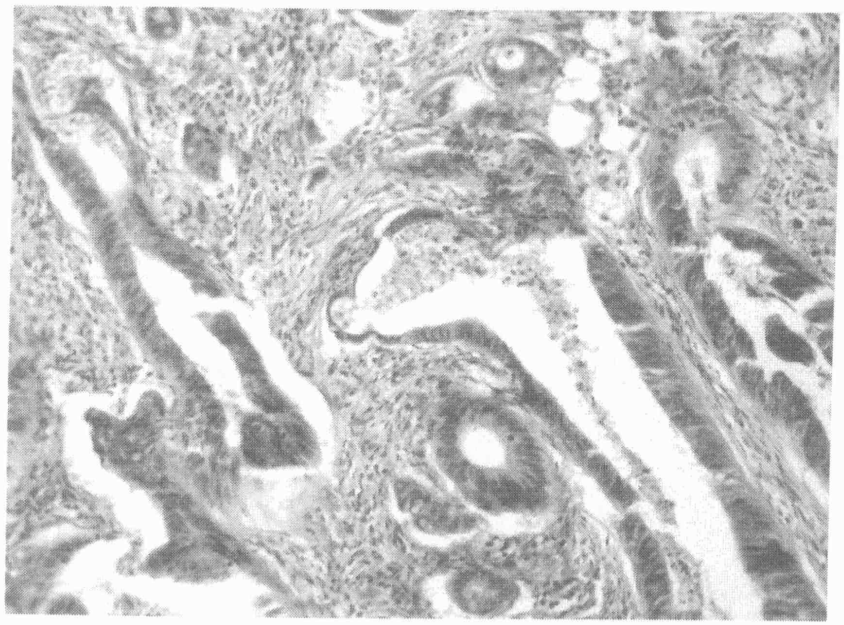
To examine the blood vessels density in CRC and relate it to levels of gene expression of $VEGF_{121}$, $VEGF_{165}$, site of tumour, Duke's stage and MSI status of the tumour.

7.3 Methods

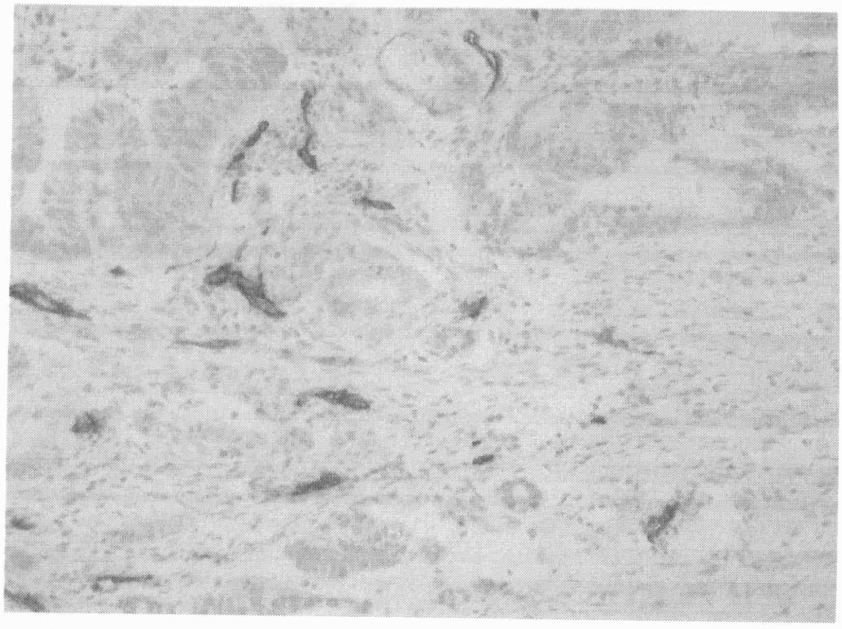
Immuno-histochemistry, of wax embedded tumour blocks, was used to measure the blood vessel density of CRC (Section 3.4.2). A monoclonal CD34 antibody was used to detect the antigen. CD34 is selectively expressed on human lymphoid and myeloid haemopoietic progenitor cells which mean it can be used to detect blood vessels. Antigen retrieval, using citrate buffer, and hydrogen peroxidase treatment was used. Avidin biotin staining technique was used and the sections were counterstained with Mayer's haematoxylin (Tables 3.9, 3.10, 3.11).

The blood vessel density, mean blood vessel density per high-power field was calculated using a 25-point Chalkley point eyepiece graticule. The hotspots were analysed at x200-250 magnification by two independent investigators both of which were blind to the characteristics of the tumours (Section 3.4.4).

A



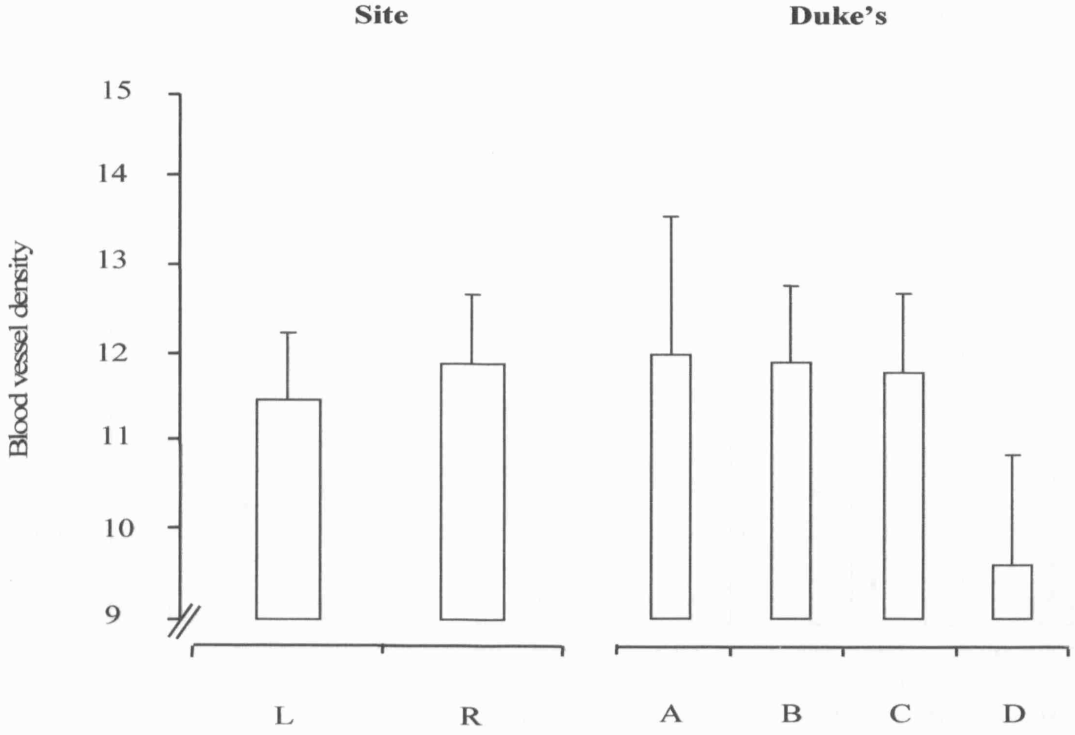
B



Picture 7.1
A is a picture of H & E staining of sample 40. **B** is a picture of staining of CD34 representing blood vessel hotspots of sample 40. Magnification x200.

7.4 Results

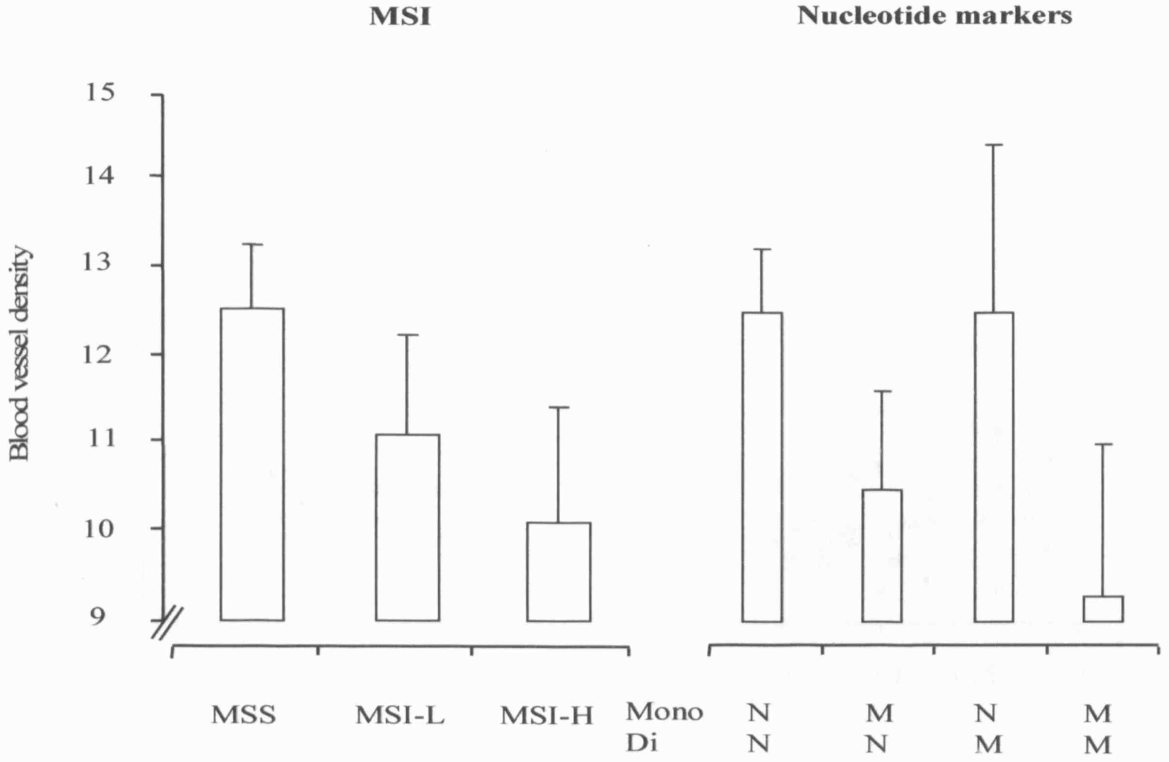
Comparing the site of tumours with blood vessel density there was no difference between right and left CRC (Figure 7.1, p>0.1, ANOVA). It was investigated to see whether there was a correlation between blood vessel density and the stage of the CRC. There was no definite correlation but there was a higher blood vessel density in Duke's A tumours compared to Duke's D (Figure 7.1, p>0.1, ANOVA).



	Site		Duke's Stage			
	Left	Right	A	B	C	D
Mean blood vessel density per high-power field	11.5	11.9	12.0	11.9	11.8	9.60
s.e.m.	0.76	0.80	1.58	0.88	0.89	1.26

Figure 7.1
Mean blood vessel per high-power field according to the site of tumour and Duke's stage. The numbers represent the mean blood vessel density per high power field and the s.em. p>0.1, ANOVA.

Blood vessel density was then analysed according to MSI status. MSS tumours had the highest blood vessel density which decreased in a stepwise fashion to MSI-H tumours (Figure 7.2, $p<0.09$, ANOVA). Further analysing the results for mutations of mononucleotide and dinucleotide markers, the highest blood vessel density was in tumours with no mutations and tumours with only mutated dinucleotide markers (Figure 7.2, $p>0.1$, ANOVA).



	MSI			Nucleotide markers			
	MSS	MSI-L	MSI-H	NN	MN	NM	MM
Mean blood vessel density per high-power field	12.5	11.1	10.1	12.5	10.5	12.5	9.29
s.e.m.	0.72	1.11	1.30	0.72	1.11	1.90	1.71

Figure 7.2
Mean blood vessel density per high-power field according to MSI and which nucleotide markers were mutated. Mono = mononucleotide markers, Di = dinucleotide markers. N – nucleotide marker not mutated, M nucleotide marker mutated. The numbers represent the mean blood vessel density per high power field and the s.em. $p>0.1$, ANOVA.

There was no correlation between $VEGF_{121}$ and $VEGF_{165}$ and blood vessel density (Figure 7.3, $p>0.05$, Pearson's Bivariate correlation).

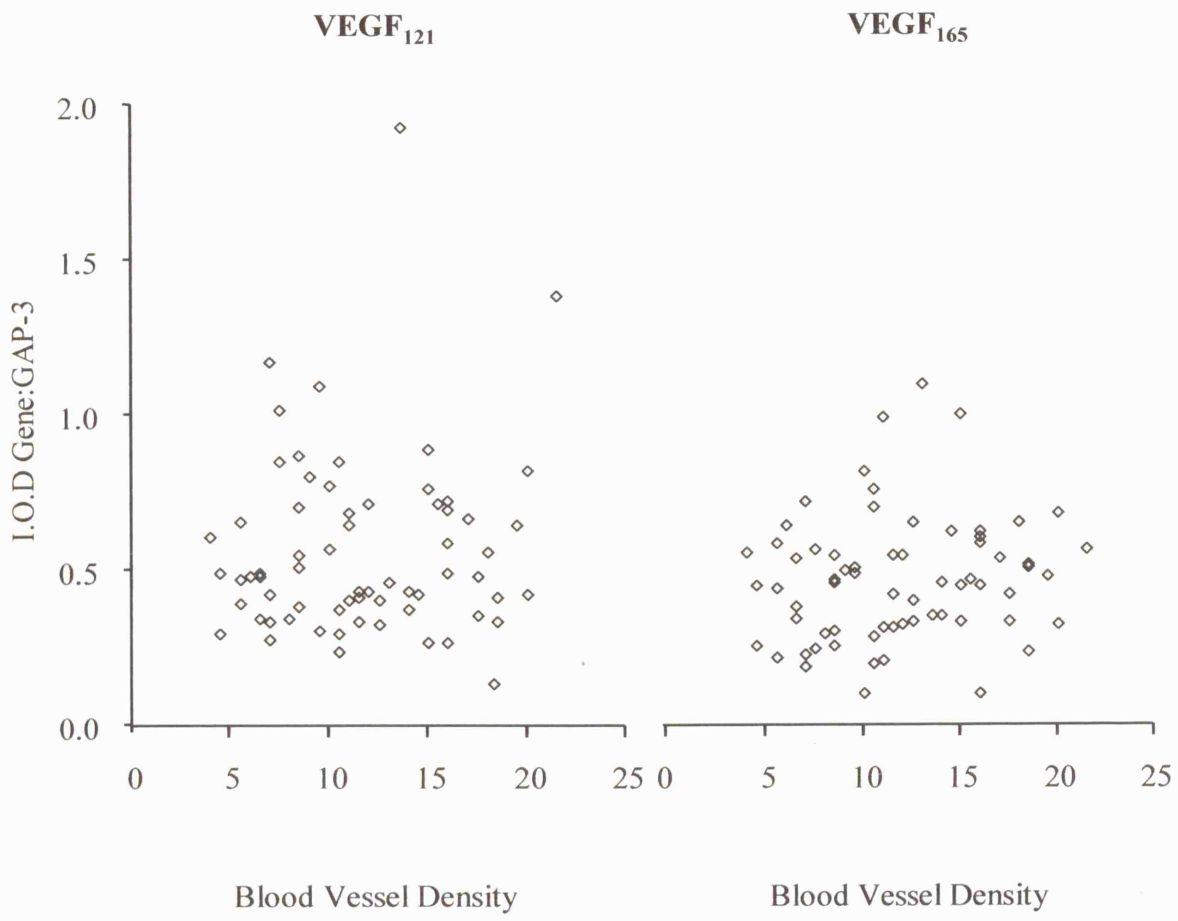


Figure 7.3
Ratio of integrated optical density (IOD) of $VEGF_{121}$ and $VEGF_{165}$ to the house keeping gene GAP-3 in CRC according to mean blood vessel density per high-power field. $p>0.1$, Pearson's Bivariate correlation.

7.5 Discussion

There was no difference in the mean blood vessel density between left and right tumours, and this does not correspond with the increased gene expression of $VEGF_{121}$ in left tumours. The lack of increase in blood vessel density in left tumours maybe due to other inhibitory factors inhibiting angiogenesis, such as p53, IL-10 or IL-13 (Mukhopadhyay *et al.*, 1995, Matsumoto *et al.*, 1998).

The highest blood vessel density was found in Duke's A tumours, with the lowest in Duke's D tumours. Although the difference is not significant the results may indicate that Duke's A tumours undergo more angiogenesis to produce more blood vessels so that tumours can grow. The highest blood vessel density in Duke's A tumours corresponds with the increased gene transcription of $VEGF_{121}$. This agrees with studies which found that the angiogenic switch in tumours is thought to occur early or during the pre- neoplastic stage (Carmeliet & Jain, 2000, Hanrahan *et al.*, 2003).

There was a decreasing trend in the number of blood vessels from MSS to MSI-H tumours. This again corresponds with a lower level of gene transcription of $VEGF_{121}$ in MSI-H compared to MSI-L tumours. This supports the evidence that $VEGF_{121}$ may stimulate angiogenesis and be involved in the formation of blood vessels in CRC. There is a larger difference in blood vessel density when the results were analysed according to which markers are mutated. Tumours with mutated dinucleotide markers had an even higher blood vessel density which corresponds with $VEGF_{121}$ transcription. The decrease in mean blood vessel density with MSI agrees with studies that found decreased angiogenesis with CRC with MSI-H (Wynter *et al.*,1999, Karnes *et al.*,

1998). The lower level of angiogenesis may be one factor which explains the improved prognosis of MSI-H tumours.

The lack of correlation between $VEGF_{121}$ or $VEGF_{165}$ and mean blood vessel density contradicts previous studies by Nakasaki *et al.* (2002) and Tsuji *et al.* (2002). However, these two studies compared $VEGF_A$ protein expression with mean blood vessel density rather than $VEGF_{121}$ and $VEGF_{165}$ transcription. Measuring $VEGF_A$ protein expression will be measuring the storage isoforms as well as the soluble isoforms. This may mean that the soluble isoforms stimulate angiogenesis but there is not a positive correlation with blood vessel density due to some of the soluble isoforms being converted to storage VEGF isoforms once the blood vessels have been formed by angiogenesis. Hence there is a correlation between $VEGF_A$ protein and blood vessel density but not with $VEGF_{121}$ and $VEGF_{165}$ transcription.

In conclusion, there was no significant evidence that an alteration in blood vessel density occurs with Duke's stage and MSI and site of the tumour. However, there are indications that there is an increased blood vessel density in early CRC and lowest in MSI-H tumours and this may reflect alterations in levels of angiogenesis. The lack of correlation between $VEGF_{121}$ and $VEGF_{165}$ and blood vessel density reflects the act that there are several factors involved in the mechanism of angiogenesis and VEGF is not the only ligand involved.

Summary

1. The highest blood vessel density was in Duke's A compared to Duke's D.
2. The highest blood vessel density was in MSS tumours compared to MSI-H tumours.
3. There was no correlation between $VEGF_{121}$ and $VEGF_{165}$ transcription and blood vessel density.

CHAPTER 8

DISCUSSION AND FINAL CONCLUSIONS

8.1 Discussion and final conclusions

The aim of this thesis was to investigate further why MSI-H tumours have a better prognosis compared to MSI-L and MSS tumours concentrating on $VEGF_{121}$, $VEGF_{165}$ and $VEGF_C$ which may confer differing angio- and lymphangiogenic potential.

The sample demographics were consistent with previous published results, with the majority of CRC being left sided, Duke's B and C, and 18% were MSI-H. The median age of the sample population was only slightly higher than previous published results. The characteristics of the samples MSI-H tumours were also in agreement with known characteristics of MSI-H tumours, with the majority being female, older and tumours were in the right side of the colon. The consistency of the demographics of the sample allowed them to be used as representative of CRC.

Wax embedded tumour blocks were used as samples for both RT-PCR and immunohistochemistry. The use of wax embedded blocks, using the techniques which have previously been described, shows they are an effective source for both gene transcription and protein analysis. The lack of correlation between gene and protein expression may be due to methodology or it reflects there are multiple pathways involved in angiogenesis and lymphogenesis and so there are several factors involved in the control of these mechanisms.

The analysis of gene transcription according to MSI showed that there was a lower level of $VEGF_{121}$ gene transcription in MSI-H tumours. To correspond with the changes in $VEGF_{121}$ gene transcription there was a trend towards an inverse correlation between blood vessel density and MSI status. The results were also given extra weight by there

being a lower level of $VEGF_{121}$ gene transcription in right sided tumours and older patients. This is in keeping with the fact that MSI-H tumours are known to occur most frequently in older female patients and are most commonly right sided tumours. The difference in the phenotype of MSI-H tumours may be explained by a decreased level of $VEGF_{121}$ which reduces the stimulus towards angiogenesis and so decrease the CRC potential to grow and spread. In addition, $VEGF_{121}$ soluble isoform appears to be involved in the process of angiogenesis in CRC, due to there being a higher level of $VEGF_{121}$ gene transcription in CRC than normal colonic mucosa.

Analysis of the data according to Duke's stage, for both $VEGF_{121}$ gene transcription and blood vessel density showed a trend towards an inverse correlation from Duke's A to Duke's D. This also reflects that $VEGF_{121}$ has a role in carcinogenesis early in the pathway towards the development of a tumour and so maybe involved in the 'angiogenic switch' towards formation of a CRC.

In contrast to $VEGF_{121}$, $VEGF_{165}$ had no obvious relationship with any of the CRC characteristics. It was found however that $VEGF_{165}$ gene transcription was decreased in CRC when compared to normal colon samples and this is an opposite finding to $VEGF_{121}$. Thus it may be suggested that in the presence of CRC $VEGF_{165}$ is down regulated to facilitate up regulation of $VEGF_{121}$. In addition to this, $VEGF_{165}$ had a positive correlation $VEGF_C$ and this may reflect that $VEGF_{165}$ also facilitates the function of $VEGF_C$.

$VEGF_C$ gene transcription had no correlation with tumour characteristics apart from MSI status. There was a significant increase in $VEGF_C$ gene transcription in true MSI-

L tumours compared to MSI-H tumours and there was in addition an increase in $VEGF_C$ protein expression although it was not significant. This may reflect that there is a decrease in lymphogenesis in MSI-H tumours which along with a decrease in angiogenesis accounts for the better prognosis of MSI-H tumours.

In contrast, to gene transcription, protein C expression was correlated with right sided CRC and the highest level of protein C expression was in Duke's D. These results reflect the fact that $VEGF_C$ maybe involved in the pathogenesis of CRC through lymphogenesis. The difference in results between gene transcription and protein expression of $VEGF_C$ may indicate that the rate controlling factors are post transcriptional or post translational.

The argument, by Jass *et al.* (2002), that true MSI-L tumours are those which only have a mutation in dinucleotide markers has been extra support by this research. The fact that that there was increased gene expression of $VEGF_{121}$ and $VEGF_C$ plus increased blood vessel density and $VEGF_C$ protein in CRC with only mutated dinucleotide markers, indicates that this group has a distinct molecular identity which may contribute to the 'true' MSI-L phenotype.

Summary

1. MSI-H tumours were mostly found in female, older patients and in right sided colon cancer.
2. Wax embedded tumour blocks are an effective source for analysis of both gene transcription and protein.
3. MSI-H tumours had a lower level of $VEGF_{121}$ gene transcription. In addition there

was a trend towards an inverse correlation with blood vessel density and MSI status.

4. There was a trend towards an inverse correlation between Duke's stage and $VEGF_{121}$ and blood vessel density.
5. True MSI-L tumours had an increased level of $VEGF_C$ gene transcription.

In summary, $VEGF_{121}$ and $VEGF_C$ appear to be involved in the pathogenesis of CRC via angiogenesis and lymphogenesis and may account towards why there is a difference in the phenotype of CRC with MSI.

Future work could include investigating further the pathways of angiogenesis and lymphogenesis and the correlation with MSI tumours. This could involve analysing MSI status according to the receptors for $VEGF_A$ and $VEGF_C$ or other genes involved in the process of angiogenesis such as COX2 or bFGF. It would also be interesting to perform a microarray analysis on CRC with MSI using a limited chip targeted at the genes involved in angiogenesis. Further experiments could also investigate why MACS tumours have a different phenotype to MSI tumours and whether they can be accounted for by differences in the levels of angiogenesis and lymphogenesis.

LIST OF PUBLICATIONS

PUBLICATIONS

1. Activity (transcription) of the genes for MLH1, MSH2 and p53 in sporadic colorectal tumours with micro-satellite instability.

Tou SIH, Drye ER, Boulos PB, Hollingsworth SJ (2004). B.J.C.,4:1-7.

2. Expression of Angiogenic VEGF-A (soluble isoforms 121, 165) and Lymphangiogenic VEGF-C in > Colorectal Cancers with Micro-satellite Instability.

Hollingsworth SJ, Drye ER, Tou SIH, Boulos PB (2005). J.Surg.Oncol.,92:317-25.

LIST OF PRESENTATIONS

PRESENTATIONS

International

The American Society of Colon and Rectal Surgeons, New Orleans, 2003

Tou S, Drye ER, Boulos PB, Hollingsworth SJ (2003). Is transcription of DNA mismatch repair genes MLH1 and MSH2 and tumour suppressor gene p53 important in colorectal cancers with microsatellite instability?

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Tou S, Drye ER, Boulos PB, Hollingsworth SJ (2003). Is transcription of DNA mismatch repair genes MLH1 and MSH2 and tumour suppressor gene p53 important in colorectal cancers with microsatellite instability?

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Drye ER, Tou S, Boulos PB, Hollingsworth SJ (2004). Elevated activity of the gene for the angiogenic $VEGF_A$ soluble isoform $VEGF_{121}$ in sporadic colorectal cancers with low-level micro-satellite instability.

Tou S, Drye ER, Boulos PB, Hollingsworth SJ (2004). Reduced activity of the tumour-suppressing gene p53 rather than mutation is important in sporadic colorectal cancers with high-level microsatellite instability.

Hospital

The Middlesex Hospital: The Pearce GouldVisiting Professor April 2003

Drye ER, Tou S, Boulos PB, Hollingsworth SJ (2003). Colorectal cancers with Microsatellite Instability: altered transcription of angiogenic $VEGF_A$ soluble isoforms and lymphogenic $VEGF_C$.

LIST OF ABSTRACTS

ABSTRACTS

Drye ER, Tou S, Boulos PB, Hollingsworth SJ (2003). Colorectal cancers with Microsatellite Instability: altered transcription of angiogenic $VEGF_A$ soluble isoforms ($VEGF_{121}$, $VEGF_{165}$) and lymphogenic $VEGF_C$. Colorectal Disease, 5S1:23-24.

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APPENDICES

APPENDIX I

Tumour characteristic, $VEGF_{121/165}$ gene transcription and blood vessel density

Sample No	Gender	Age (years)	Site	Dukes'	MSI	$VEGF_{121}$			$VEGF_{165}$			Blood vessel density		
						IOD 1	IOD 2	Mean	IOD 1	IOD 2	Mean	Count1	Count2	Mean
1	Male	38	Left	C	Low	0.445	0.740	0.593	0.555	0.531	0.543	4	4	4
2	Male	62	Left	B	Low	1.330	0.985	1.157	0.092	0.330	0.211	7	7	7
3	Male	77	Left	C	Stable	0.500	0.215	0.358	0.953	0.546	0.750	10	11	10.5
4	Female	65	Left	B	Stable	0.690	0.892	0.791	0.552	0.426	0.489	10	8	9
6	Male	72	Left	B	Stable	0.592	0.336	0.791	0.698	0.561	0.629	7	5	6
9	Male	75	Left	B	Low	0.439	0.345	0.377	0.594	0.352	0.432	6	5	5.5
11	Female	90	Left	B	Stable	0.451	0.191	0.321	0.625	0.805	0.715	8	6	7
12	Male	53	Left	B	Stable	0.646	0.191	0.633	0.604	0.328	0.466	17	22	19.5
13	Male	87	Left	C	Stable	1.021	0.208	0.479	0.674	0.214	0.367	7	6	6.5
14	Male	71	Left	B	Stable	0.566	0.249	0.408	0.918	0.498	0.708	7	7	7
16	Female	66	Left	B	Stable	0.620	0.197	0.408	0.297	0.319	0.308	22	18	20
17	Female	82	Left	C	Stable	0.619	0.282	0.451	1.301	0.885	1.093	12	14	13
18	Male	66	Left	A	Low	0.981	1.178	1.080	0.371	0.583	0.477	11	8	9.5

Sample No	Gender	Age (years)	Site	Dukes'	MSI	$VEGF_{121}$			$VEGF_{165}$			Blood vessel density		
						IOD 1	IOD 2	Mean	IOD 1	IOD 2	Mean	Count1	Count2	Mean
21	Female	73	Left	C	High	0.584	0.381	0.482	0.787	0.097	0.442	4	5	4.5
24	Female	72	Left	D	Stable	0.535	0.252	0.393	0.278	0.320	0.299	9	13	11
25	Female	69	Left	B	Stable	2.747	1.069	1.908	0.069	0.612	0.341	13	14	13.5
26	Female	75	Right	C	Stable	1.131	0.488	0.810	1.239	0.111	0.675	22	18	19.5
27	Female	89	Left	D	High	0.675	0.100	0.387	0.766	0.531	0.649	12	13	12.5
28	Male	85	Left	C	Low	0.238	0.403	0.321	0.774	0.225	0.499	22	15	18.5
29	Male	66	Left	B	Low	1.061	0.440	0.751	0.650	0.228	0.439	16	14	15
30	Male	100	Right	C	Stable	0.590	0.250	0.363	0.770	0.290	0.449	12	16	14
31	Male	73	Right	C	Stable	0.781	0.332	0.577	1.117	0.493	0.805	10	10	10
32	Female	83	Right	B	Stable	0.440	0.346	0.393	0.411	0.370	0.390	9	16	12.5
33	Female	82	Left	C	Stable	0.364	0.206	0.285	1.006	0.125	0.188	11	10	11.5
35	Male	78	Right	B	Stable	0.761	0.197	0.479	1.006	0.125	0.572	14	18	16
36	Male	74	Left	D	Stable	0.819	0.102	0.461	0.831	0.315	0.573	5	6	5.5

Sample No	Gender	Age (years)	Site	Dukes'	MSI	$VEGF_{121}$			$VEGF_{165}$			Blood vessel density		
						IOD 1	IOD 2	Mean	IOD 1	IOD 2	Mean	Count1	Count2	Mean
37	Male	68	Right	B	Low	0.456	0.223	0.340	0.380	0.431	0.405	17	18	17.5
38	Male	68	Right	B	Stable	0.160	0.092	0.126	0.811	0.239	0.508	17	20	18.5
39	Female	81	Right	B	High	0.833	0.255	0.544	0.529	0.759	0.644	16	20	18
40	Male	90	Left	C	Stable	1.123	0.182	0.652	0.811	0.239	0.525	17	17	17
41	Female	80	Left	C	Stable	0.695	0.129	0.412	0.635	0.591	0.613	15	14	14.5
42	Male	64	Right	B	Stable	0.660	0.336	0.498	0.696	0.200	0.448	9	8	8.5
44	Male	68	Left	B	Stable	0.843	0.584	0.713	0.551	0.325	0.438	16	16	16
45	Male	86	Left	A	Stable	0.599	0.239	0.419	0.502	0.180	0.341	15	13	14
47	Female	53	Left	B	High	0.406	0.886	0.646	0.122	0.280	0.201	5	6	5.5
49	Male	58	Right	C	Stable	0.707	0.496	0.534	0.426	0.045	0.292	6	11	8.5
50	Male	60	Right	B	Stable	0.307	0.263	0.285	0.501	0.494	0.248	5	4	4.5
51	Female	77	Right	C	Low	0.688	0.110	0.399	0.961	0.106	0.533	12	11	11.5
55	Male	70	Left	B	Low	0.410	0.240	0.325	0.529	0.083	0.306	12	11	11.5

Sample No	Gender	Age (years)	Site	Dukes'	MSI	$VEGF_{121}$			$VEGF_{165}$			Blood vessel density		
						IOD 1	IOD 2	Mean	IOD 1	IOD 2	Mean	Count1	Count2	Mean
56	Female	77	Right	C	Stable	0.360	0.139	0.249	0.369	0.283	0.326	14	16	15
57	Male	77	Left	C	Stable	0.279	0.225	0.252	0.096	0.088	0.092	16	16	16
58	Male	80	Right	C	Low	0.990	1.018	1.004	0.034	1.073	0.554	8	7	7.5
61	Female	68	Right	B	High	0.323	0.514	0.418	0.501	0.572	0.537	11	13	12
63	Female	88	Right	C	High	0.336	0.280	0.308	0.451	0.193	0.322	13	12	12.5
64	Female	82	Right	C	High	0.405	0.532	0.468	0.452	0.188	0.320	19	16	17.5
66	Female	67	Left	A	Stable	0.720	0.958	0.839	0.046	0.425	0.236	6	9	7.5
67	Male	53	Right	B	Low	0.951	0.443	0.697	0.234	0.245	0.240	7	10	8.5
70	Male	71	Right	B	High	0.244	0.416	0.330	0.347	0.697	0.522	7	6	6.5
71	Female	69	Right	B	High	0.497	0.238	0.330	0.830	0.253	0.522	8	9	8.5
72	Female	59	Left	B	Stable	0.652	0.501	0.577	0.959	0.269	0.614	17	15	16
73	Female	54	Right	B	Low	0.404	0.183	0.294	0.667	0.326	0.496	9	10	9.5
74	Female	80	Left	B	Low	1.339	1.408	1.374	0.338	0.777	0.557	23	20	21.5

Sample No	Gender	Age (years)	Site	Dukes'	MSI	$VEGF_{121}$			$VEGF_{165}$			Blood vessel density		
						IOD 1	IOD 2	Mean	IOD 1	IOD 2	Mean	Count1	Count2	Mean
75	Female	85	Left	B	Stable	0.910	0.488	0.699	0.668	0.240	0.454	17	14	15.5
83	Female	73	Right	C	Low	1.526	0.222	0.874	1.511	0.470	0.990	16	14	15
84	Female	89	Left	B	Low	0.749	0.766	0.757	0.015	0.155	0.085	11	9	10
85	Male	75	Left	A	Stable	0.587	0.808	0.698	0.015	0.611	0.313	12	12	12
87	Male	69	Left	A	Stable	0.703	0.972	0.838	0.553	0.832	0.693	11	10	10.5
88	Male	51	Left	D	Low	0.539	0.134	0.337	0.423	0.135	0.279	9	7	8
91	Male	72	Right	B	High	0.374	0.158	0.266	0.233	0.115	0.174	7	7	7
93	Male	57	Right	B	Stable	0.575	0.789	0.682	0.534	0.652	0.593	17	15	16
94	Female	80	Right	B	High	0.327	0.119	0.223	0.477	0.063	0.270	8	13	10.5
95	Female	61	Left	D	Low	0.670	0.682	0.676	0.080	0.318	0.199	12	10	11
96	Female	68	Right	C	High	0.601	0.340	0.471	0.483	0.178	0.331	7	6	6.5
97	Male	49	Right	C	Stable	0.806	0.456	0.631	0.617	1.353	0.985	12	10	11
98	Male	38	Left	C	Low	0.700	1.018	0.859	0.521	0.395	0.458	9	8	8.5

Sample No	Gender	Age (years)	Site	Dukes'	MSI	$VEGF_{121}$			$VEGF_{165}$			Blood vessel density		
						IOD 1	IOD 2	Mean	IOD 1	IOD 2	Mean	Count1	Count2	Mean
100	Female	91	Left	A	Stable	0.465	0.337	0.401	0.224	0.218	0.221	17	20	18.5
102	Female	79	Right	C	Stable	0.383	0.457	0.420	0.268	0.549	0.408	13	10	11.5
C0033	Male	57		T.A		0.042	0.727	0.384	0.313	1.017	0.665			
C0035	Female	71		A		0.044	0.380	0.212	0.364	1.175	0.770			
C0036	Female	87		B		0.064	0.142	0.103	0.561	0.201	0.381			
C0037	Male	72		C		0.091	0.455	0.273	0.498	0.913	0.706			
C0038	Female	45		B		0.212	0.766	0.489	0.667	0.445	0.556			
C0039	Male	64		B		0.339	0.431	0.385	0.554	0.484	0.519			
C0040	Male	66		A		0.380	0.412	0.396	0.851	0.429	0.640			
C0041	Male	82		B		0.222	0.582	0.402	0.495	0.495	0.495			

APPENDIX II

Tumour characteristic, $VEGF_C$ gene transcription and protein C expression

Sample No	Gender	Age (years)	Site	Dukes'	MSI	$VEGF_{CI}$			$VEGF_{CII}$			Mean Total $VEGF_C$	% Protein C expression		
						IOD 1	IOD 2	Mean	IOD 1	IOD 2	Mean		Count1	Count2	Mean
1	Male	38	Left	C	Low	0.452	0.853	0.652	0.610	0.678	0.644	0.648	80	90	85
2	Male	62	Left	B	Low	0.230	0.249	0.239	0.350	0.388	0.369	0.304	80	80	80
3	Male	77	Left	C	Stable	0.090	0.287	0.188	0.351	0.434	0.393	0.291	60	70	65
4	Female	65	Left	B	Stable	0.093	0.702	0.397	0.294	0.616	0.455	0.426	90	90	90
6	Male	72	Left	B	Stable	0.046	0.460	0.253	0.322	1.044	0.683	0.468	80	80	80
9	Male	75	Left	B	Low	0.464	0.365	0.397	0.905	0.770	0.816	0.607	80	80	80
11	Female	90	Left	B	Stable	0.723	0.341	0.532	0.772	0.718	0.745	0.639	60	40	50
12	Male	53	Left	B	Stable	0.265	0.362	0.131	0.299	0.356	0.327	0.320	5	10	7.5
13	Male	87	Left	C	Stable	0.147	0.341	0.276	0.942	0.892	0.908	0.592	90	90	90
14	Male	71	Left	B	Stable	0.332	0.194	0.263	0.531	0.439	0.485	0.374	60	70	65
16	Female	66	Left	B	Stable	0.490	0.209	0.350	0.958	0.369	0.664	0.507	50	60	55
17	Female	82	Left	C	Stable	0.485	0.365	0.425	0.767	0.542	0.654	0.540	50	50	50
18	Male	66	Left	A	Low	0.213	0.312	0.263	0.331	0.627	0.479	0.371	40	60	50

Sample No	Gender	Age (years)	Site	Dukes'	MSI	$VEGF_{CI}$			$VEGF_{CII}$			Mean Total $VEGF_C$	% Protein C expression		
						IOD 1	IOD 2	Mean	IOD 1	IOD 2	Mean		Count1	Count2	Mean
21	Female	73	Left	C	High	0.409	0.314	0.361	0.752	0.979	0.866	0.614	80	60	70
24	Female	72	Left	D	Stable	0.326	0.233	0.280	0.915	0.553	0.734	0.507	50	70	60
25	Female	69	Left	B	Stable	0.370	0.326	0.348	0.314	0.673	0.494	0.421	80	60	70
26	Female	75	Right	C	Stable	0.360	0.200	0.280	0.548	0.182	0.365	0.322	80	80	80
27	Female	89	Left	D	High	0.317	0.187	0.252	0.669	0.777	0.723	0.487	70	80	75
28	Male	85	Left	C	Low	0.216	0.327	0.272	1001	0.494	0.753	0.512	60	60	60
29	Male	66	Left	B	Low	0.622	0.359	0.490	1.550	1.066	1.308	0.899	90	90	90
30	Male	100	Right	C	Stable	0.360	0.364	0.363	0.601	0.947	0.832	0.597	80	80	80
31	Male	73	Right	C	Stable	0.603	0.389	0.496	0.978	1.011	0.904	0.700	70	40	55
32	Female	83	Right	B	Stable	0.151	0.291	0.211	0.615	0.836	0.725	0.473	80	80	80
33	Female	82	Left	C	Stable	0.197	0.282	0.240	0.570	0.376	0.473	0.356	80	80	80
35	Male	78	Right	B	Stable	0.248	0.282	0.201	0.492	0.316	404	0.303	80	90	85
36	Male	74	Left	D	Stable	0.689	0.395	0.542	0.876	0.415	0.645	0.594	90	90	90

Sample No	Gender	Age (years)	Site	Dukes'	MSI	$VEGF_{CI}$			$VEGF_{CII}$			Mean Total $VEGF_C$	% Protein C expression		
						IOD 1	IOD 2	Mean	IOD 1	IOD 2	Mean		Count1	Count2	Mean
37	Male	68	Right	B	Low	0.133	0.833	0.627	0.544	0.870	0.707	0.667	80	90	85
38	Male	68	Right	B	Stable	0.568	0.661	0.397	0.556	0545	0.551	0.474	70	70	70
39	Female	81	Right	B	High	0.788	0.311	0.440	0.722	0.595	0.659	0.549	90	90	90
40	Male	90	Left	C	Stable	0.483	0.195	0.491	1.075	0.676	0.876	0.684	90	90	90
41	Female	80	Left	C	Stable	0.483	0.165	0.324	0.808	0.225	0.517	0.420	90	90	90
42	Male	64	Right	B	Stable	0.204	0.215	0.209	0.408	0.269	0.339	0.274	80	90	85
44	Male	68	Left	B	Stable	0.268	0.439	0.353	0.393	0.376	0.385	0.369	90	90	90
45	Male	86	Left	A	Stable	0.636	0.304	0.470	0.528	0.389	0.583	0.526	5	10	7.5
47	Female	53	Left	B	High	0.112	0.521	0.317	0.472	0.465	0.469	0.393	50	40	45
49	Male	58	Right	C	Stable	0.476	0.087	0.218	0.620	0.328	0.470	0.376	70	80	75
50	Male	60	Right	B	Stable	0.602	0.118	0.360	0.388	0.662	0.525	0.442	80	80	80
51	Female	77	Right	C	Low	0.556	0.301	0.429	0.695	0.093	0.394	0.411	90	90	90
55	Male	70	Left	B	Low	0.287	0.359	0.323	0.842	0.639	0.740	0.532	90	90	90

Sample No	Gender	Age (years)	Site	Dukes'	MSI	$VEGF_{CI}$			$VEGF_{CII}$			Mean Total $VEGF_C$	% Protein C expression		
						IOD 1	IOD 2	Mean	IOD 1	IOD 2	Mean		Count1	Count2	Mean
56	Female	77	Right	C	Stable	0.368	0.186	0.277	0.765	0.522	0.643	0.460	90	90	90
57	Male	77	Left	C	Stable	0.407	0.112	0.259	0.748	0.553	0.650	0.455	90	80	85
58	Male	80	Right	C	Low	0.295	0.183	0.239	0.776	1.505	1.140	0.690	90	80	85
61	Female	68	Right	B	High	0.237	0.399	0.318	0.447	0.725	0.586	0.452	70	70	70
63	Female	88	Right	C	High	0.194	0.189	0.192	0.373	0.378	0.376	0.284	90	80	85
64	Female	82	Right	C	High	0.167	0.048	0.108	0.303	0.622	0.463	0.285	70	70	70
66	Female	67	Left	A	Stable	0.200	0.172	0.186	1.133	1.598	1.365	0.776	80	90	85
67	Male	53	Right	B	Low	0.571	0.128	0.350	1.318	0.380	0.736	0.543	80	90	85
70	Male	71	Right	B	High	0.321	0.149	0.235	0.499	0.541	0.520	0.378	90	90	90
71	Female	69	Right	B	High	0.503	0.388	0.446	1.362	0.414	0.888	0.667	90	90	90
72	Female	59	Left	B	Stable	0.721	0.568	0.645	0.850	1.062	0.956	0.800	90	90	90
73	Female	54	Right	B	Low	0.564	0.429	0.496	0.971	0.559	0.765	0.631	90	90	90
74	Female	80	Left	B	Low	0.274	0.235	0.255	0.991	1.049	1.020	0.637	90	80	85

Sample No	Gender	Age (years)	Site	Dukes'	MSI	$VEGF_{CI}$			$VEGF_{CII}$			Mean Total $VEGF_C$	% Protein C expression		
						IOD 1	IOD 2	Mean	IOD 1	IOD 2	Mean		Count1	Count2	Mean
75	Female	85	Left	B	Stable	0.410	0.335	0.373	0.850	0.382	0.389	0.381	90	90	90
83	Female	73	Right	C	Low	0.759	0.521	0.640	0.912	0.438	0.675	0.658	70	70	70
84	Female	89	Left	B	Low	0.134	0.339	0.236	0.655	0.736	0.695	0.466	70	80	75
85	Male	75	Left	A	Stable	0.085	0.252	0.168	0.613	0.994	0.803	0.486	60	60	60
87	Male	69	Left	A	Stable	0.103	0.261	0.182	0.737	1.028	0.882	0.532	60	70	65
88	Male	51	Left	D	Low	0.107	0.545	0.326	0.508	0.425	0.466	0.396	90	90	90
91	Male	72	Right	B	High	0.185	0.067	0.126	1.755	0.417	0.949	0.538	90	90	90
93	Male	57	Right	B	Stable	0.074	0.183	0.129	0.343	0.554	0.449	0.289	90	90	90
94	Female	80	Right	B	High	0.227	0.544	0.385	0.806	0.608	0.707	0.546	90	90	90
95	Female	61	Left	D	Low	0.183	0.058	0.120	0.406	0.695	0.550	0.335	90	90	90
96	Female	68	Right	C	High	0.233	0.337	0.285	0.655	0.601	0.628	0.457	90	90	90
97	Male	49	Right	C	Stable	0.077	0.142	0.109	0.635	1.451	1.043	0.576	90	90	90
98	Male	38	Left	C	Low	0.257	0.128	0.192	0.762	0.678	0.720	0.456	90	90	90

Sample No	Gender	Age (years)	Site	Dukes'	MSI	$VEGF_{CI}$			$VEGF_{CII}$			Mean Total $VEGF_C$	% Protein C expression		
						IOD 1	IOD 2	Mean	IOD 1	IOD 2	Mean		Count1	Count2	Mean
56	Female	77	Right	C	Stable	0.368	0.186	0.277	0.765	0.522	0.643	0.460	90	90	90
57	Male	77	Left	C	Stable	0.407	0.112	0.259	0.748	0.553	0.650	0.455	90	80	85
58	Male	80	Right	C	Low	0.295	0.183	0.239	0.776	1.505	1.140	0.690	90	80	85
61	Female	68	Right	B	High	0.237	0.399	0.318	0.447	0.725	0.586	0.452	70	70	70
63	Female	88	Right	C	High	0.194	0.189	0.192	0.373	0.378	0.376	0.284	90	80	85
64	Female	82	Right	C	High	0.167	0.048	0.108	0.303	0.622	0.463	0.285	70	70	70
66	Female	67	Left	A	Stable	0.200	0.172	0.186	1.133	1.598	1.365	0.776	80	90	85
67	Male	53	Right	B	Low	0.571	0.128	0.350	1.318	0.380	0.736	0.543	80	90	85
70	Male	71	Right	B	High	0.321	0.149	0.235	0.499	0.541	0.520	0.378	90	90	90
71	Female	69	Right	B	High	0.503	0.388	0.446	1.362	0.414	0.888	0.667	90	90	90
72	Female	59	Left	B	Stable	0.721	0.568	0.645	0.850	1.062	0.956	0.800	90	90	90
73	Female	54	Right	B	Low	0.564	0.429	0.496	0.971	0.559	0.765	0.631	90	90	90
74	Female	80	Left	B	Low	0.274	0.235	0.255	0.991	1.049	1.020	0.637	90	80	85

Sample No	**Gender**	**Age** (years)	**Site**	**Dukes'**	**MSI**	**$VEGF_{CI}$**			**$VEGF_{CII}$**			**Mean Total $VEGF_C$**	**% Protein C expression**		
						IOD 1	IOD 2	Mean	IOD 1	IOD 2	Mean		Count1	Count2	Mean
100	Female	91	Left	A	Stable	0.215	0.112	0.164	0.405	0.605	0.505	0.334	90	90	90
102	Female	79	Right	C	Stable	0.200	0.247	0.223	0.471	0.930	0.701	0.462	90	90	90
C0033	Male	57		T.A		0.137	0.435	0.286	0.320	1.331	0.825	0.556			
C0035	Female	71		A		0.270	0.670	0.318	0.468	1.122	0.795	0.557			
C0036	Female	87		B		0.336	0.119	0.227	0.691	0.175	0.433	0.330			
C0037	Male	72		C		0.279	0.311	0.295	0.682	0.445	0.564	0.429			
C0038	Female	45		B		0.216	0.247	0.231	1.190	0.891	1.040	0.636			
C0039	Male	64		B		0.268	0.346	0.846	0.727	0.966	0.846	0.577			
C0040	Male	66		A		0.508	0.206	0.357	0.794	0.600	0.697	0.527			
C0041	Male	82		B		0.210	0.333	0.271	0.848	0.657	0.752	0.512			